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Urine Testing and Abuse Patterns of Drugs and New Psychoactive Substances — Application of Comprehensive Time-of-Flight Mass Spectrometry



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URINE TESTING AND ABUSE PATTERNS OF DRUGS AND NEW PSYCHOACTIVE SUBSTANCES

APPLICATION OF COMPREHENSIVE
TIME-OF-FLIGHT MASS SPECTROMETRY

Mira Sundström

ACADEMIC DISSERTATION

To be presented, with the permission of the Medical Faculty of
the University of Helsinki, for public examination in the main auditorium of the
Institute of Dentistry, Mannerheimintie 172,
on 20 October 2017, at 12:00 noon.

Helsinki 2017

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**Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam
Universitatis Helsinkiensis No. 49/2017**

Cover layout by Anita Tienhaara
Cover photo by Daniel Backström

ISBN 978-951-51-3566-7 (Paperback)
ISBN 978-951-51-3567-4 (PDF)
ISSN 2342-3161 (Print)
ISSN 2342-317X (Online)
<http://ethesis.helsinki.fi>

Hansaprint
Turenki 2017

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following five articles, referred to in the text by Roman numerals **I** through **V**:

- I** Sundström M, Pelander A, Angerer V, Hutter M, Kneisel S, Ojanperä I. *A high-sensitivity ultra-high performance liquid chromatography/high-resolution time-of-flight mass spectrometry (UHPLC-HR-TOFMS) method for screening synthetic cannabinoids and other drugs of abuse in urine*, Analytical and Bioanalytical Chemistry **2013**: 405 (26); 8463–8474.
- II** Sundström M, Pelander A, Ojanperä I. *Comparison of post-targeted and pre-targeted urine drug screening by UHPLC-HR-QTOFMS*, Journal of Analytical Toxicology **2017**: 41 (1); 623–630.
- III** Sundström M, Pelander A, Ojanperä I. *Comparison between drug screening by immunoassay and ultra-high performance liquid chromatography/high-resolution time-of-flight mass spectrometry in post-mortem urine*, Drug Testing and Analysis **2015**: 7 (5); 420–427.
- IV** Sundström M, Pelander A, Simojoki K, Ojanperä I. *Patterns of drug abuse among drug users with regular and irregular attendance for treatment as detected by comprehensive UHPLC-HR-TOFMS*, Drug Testing and Analysis **2016**: 8 (1); 39–45.
- V** Heikman P, Sundström M, Pelander A, Ojanperä I. *New psychoactive substances as part of polydrug abuse within opioid maintenance treatment revealed by comprehensive high-resolution mass spectrometric urine drug screening*, Human Psychopharmacology: Clinical and Experimental **2016**: 31 (1); 44–52.

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ABBREVIATIONS

2-DPMP	2-Diphenylmethylpiperidine (desoxypipradrol)
5-IT	5-(2-aminopropyl)indole
alpha-PVP	Alpha-pyrrolidinovalerophenone
bbCID	Broadband collision-induced dissociation
CE	Collision energy
CID	Collision-induced dissociation
DAT/SERT	Dopamine/serotonin transporter
DDA	Data-dependent acquisition
DIA	Data-independent acquisition
DUID	Driving under the influence of drugs
ELISA	Enzyme-linked immunosorbent assay
EMIT	Enzyme multiplied immunoassay technique
ESI	Electrospray ionization
FN	False negative
FP	False positive
FT-ICR	Fourier-transform ion-cyclotron resonance
FWHM	Full width at half maximum
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
HEIA	Homogeneous enzyme immunoassay
HR	High resolution
LC	Liquid chromatography
LOI	Limit of identification
LR	Low-resolution
MDMA	Methylenedioxymethamphetamine
MDPV	Methylenedioxypyrovalerone
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
NMDA	N-methyl-D-aspartate
NPS	New psychoactive substance(s)
PMMA	Para-methoxymethamphetamine
Q	Quadrupole
RT	Retention time
SWATH	Sequential windowed acquisition of all theoretical mass spectra
THC-COOH	11-Nor-9-carboxy- Δ^9 -tetrahydrocannabinol
TN	True negative
TOF	Time-of-flight
TP	True positive
UHPLC	Ultra-high performance liquid chromatography

ABSTRACT

In Finland, the number of problem opioid and amphetamine users lies between 18,000 and 30,000, whereby only 20% receive treatment for their dependence. Annually illicit or medicinal drugs are found in the blood samples of more than 6,000 drivers, and approximately 500 fatal poisonings result from drugs. Drug testing attempts to unravel the role of psychoactive substances—whether used for therapeutic purposes or abused—under various circumstances. Such occasions requiring drug testing include treatment for drug users and poisoned patients, patient compliance, driving under the influence of drugs, and post-mortem toxicology. Although various techniques are available for the determination of a range of psychoactive substances, few methods provide cost-efficient means to detect the wide range of abused drugs. Many conventional methods remain too narrow in scope, complicated, or lead to false-positive or false-negative results.

A current challenge for drug testing laboratories is the wide and changing panel of abused drugs. New psychoactive substances (NPS) continually emerge on the drug scene, requiring analytical methods not necessary ten years ago. These chemically diverse substances typically remain undetected by common drug testing methods, whereby reference standards are often insufficiently available further impeding method development. NPS are manufactured predominantly in the Far East and intended to mimic the effects of conventional drugs, while circumventing narcotic laws and drug testing. Due to their rapid appearance and disappearance, NPS display a challenging group of analytes to detect.

This thesis focuses on three main objectives: first, to develop and validate a multi-analyte urine drug testing method exhibiting a high sensitivity and a high substance identification power; second, to compare the new method to two established approaches for drug testing; and third, to apply the new method to urine samples from drug abusers to assess drug abuse patterns in various groups of patients. Special attention is paid to the detection and prevalence of NPS.

A comprehensive drug testing method based on ultra-high performance liquid chromatography/high-resolution quadrupole time-of-flight mass spectrometry (UHPLC-HR-QTOFMS) in the positive ionization mode was developed for hundreds of drugs. The coverage of the method appeared more extensive than for most previous methods, enabling sensitive and cost-effective drug testing in a single analytical run from a single urine sample. Due to the utilization of mass fragmentation, isotopic pattern, and metabolite pattern, the method produced testing results with an identification reliability comparable to dedicated target analysis. In addition, due to the nontargeted acquisition of full mass range broadband data, retrospective data mining and tentative identification of compounds without a reference standard was possible.

Using the developed method, approximately one hundred drugs were identified on a routine basis including multiple NPS. Automated data processing allowed for the straightforward interpretation of results without requiring an experienced

analyst. The method outperformed immunoassay drug testing as well as a pretargeted QTOFMS approach performed on the same platform in terms of specificity, sensitivity, and scope. The limit of identification (LOI) in urine remained at a low ng/mL level for the majority of drugs. For example, LOI for buprenorphine and methamphetamine reached 1 ng/mL and 6.5 ng/mL, respectively. Cannabis, on the other hand, exhibited a higher but acceptable LOI at 15 ng/mL in the positive mode. For some NPS, subnanogram limits were reached.

The study of drug abuse patterns revealed a high prevalence of multiple drug abuse among all study subjects regardless of their treatment status. In particular, it was common among those subjects not routinely attending medical treatment for their drug problem. The most common set of abused substances consisted of buprenorphine, benzodiazepines, amphetamine, and cannabis, although subjects receiving opioid maintenance treatment more rarely abused buprenorphine. Other drugs of abuse always accompanied NPS findings, and NPS were most commonly identified in samples from individuals not receiving treatment. The analytically confirmed drug profiles of problematic Finnish drug users are reported here for the first time with a substantial accuracy, and the findings suggest that treatment for drug dependence helps reduce drug abuse. It is anticipated that the urine drug testing method presented in this thesis will find additional applications in future forensic and clinical toxicology contexts.

1 INTRODUCTION

Drug testing plays a vital role in forensic and clinical toxicology. Forensic toxicology answers questions with judicial implications involving cause of death investigations, driving under the influence of drugs, drug-facilitated crimes, and workplace drug testing. The most important areas of clinical toxicology related to drug testing consist of emergency toxicology and the treatment of drug abusers. The current trend in drug testing employs analytical techniques based on mass spectrometry (MS). In particular, high-resolution mass spectrometry (HRMS) provides advantages in terms of its sensitivity, selectivity, and flexibility, and consequently is becoming the most appealing approach to drug testing [1].

In Finland, multiple substance abuse is frequent and often involves intravenously administered buprenorphine or amphetamine [2,3]. Annually, more than 6,000 samples from apprehended drivers reveal illicit or medicinal drugs and approximately 500 fatal poisonings result from drugs. Recently, new psychoactive substances (NPS) appeared on the recreational drugs market. These drugs have effects resembling those of conventional drugs, while exhibiting higher potencies than their controlled counterparts. However, little is known about their metabolism and toxicokinetics. In response to scheduling efforts, new variants continuously emerge. Due to the transient nature of NPS, they frequently escape both typical drug screening methods and legal restrictions. During the last five years, each week two new drug variants have been launched on the European drugs market [4]. At present, over 620 NPS are being monitored across the European Union [5].

The NPS phenomenon poses challenges not only for drug testing laboratories but also for health-care services, as well as drug users themselves. Conventional drug testing methods rely on predetermined reference data obtained from commercially available reference standards. Due to the expanding repertoire of NPS, new reference standards slowly become available, thus complicating the development of NPS identification methods. Within emergency toxicology, clinicians face difficulties related to the scarcity of data on treatment strategies for NPS intoxications. In addition, NPS users are often unaware of the exact drug they are consuming, thereby increasing the risk of dosing errors and subsequent overdoses. The abuse of drugs, including NPS, will obviously become more prevalent [6], giving rise to an increased demand for drug-related health-care services.

An ideal drug screening method would cover all abused substances, whether known or unknown at the time of analysis. It is hypothesized that among available techniques HRMS is best suited for the detection of hundreds of drugs and metabolites at ng/mL concentrations in biological samples. In addition to its sensitivity and selectivity, other advantages of HRMS include nontargeted data acquisition, retrospective data mining of substances unanticipated at the time of analysis, and an easily extendable scope for new analytes. Such features are

particularly attractive in the detection of NPS displaying back and forth movements across drugs markets.

The ultimate goal of any drug screening method is to enable coverage of the entire repertoire of abused drugs, encompassing conventional drugs of abuse, prescription drugs, and transient NPS. Moreover, rarely occurring analytes such as psilocin should not be neglected [7]. However, the development of a multidrug screening method is not straightforward. Sample preparation should be suitable for a wide range of lipophilicities and molecular masses. In addition, chromatographic separation should cover early-eluting polar compounds while keeping retention times (RT) moderate for non-polar drugs as well. HRMS data should be acquired without analyte preselection, yet offer a high identification power. If all of these requirements are met, comprehensive drug testing should provide reliable patient-related and epidemiological data at a level that limited drug testing or various surveys cannot currently reach. In particular, analytically confirmed drug profiles are important in the treatment of drug users. When accurate patterns of drug abuse are revealed, treating clinicians can better instruct the drug users and prevent hazardous multiple substance abuse, potentially leading to somatic and psychiatric emergencies or even fatalities.

Consequently, today's drug testing requires a broad-spectrum method at a scope beyond conventional targeted analyses. This thesis describes a confirmation-level method based on HRMS, using ultra-high performance liquid chromatography-high-resolution quadrupole time-of-flight mass spectrometry (UHPLC-HR-QTOFMS). The developed method is one of the first multi-analyte drug screening methods that in addition to conventional drugs of abuse covers a variety of NPS, including the urinary metabolites of synthetic cannabinoids. The broad-spectrum method is applied to gathering information about the epidemiology and reasons for drug abuse through the testing of urine samples from various groups of patients.

2 REVIEW OF THE LITERATURE

2.1 Drug abuse

Substances that cause addiction induce pleasant states or relieve distress. Their continued use induces adaptive changes to the central nervous system resulting in physical or psychological dependence [8]. According to the 10th edition of the International Classification of Diseases, a diagnosis of substance dependence includes at least three of the following: a strong desire to use the substance, difficulties in controlling substance taking-behavior, symptoms of withdrawal or tolerance, a reduction in alternative pleasures or interests due to substance use, and continued substance use despite evidence of harmful consequences [9].

Moreover, multiple substance abuse can result in an increased reinforcement effect [10]. Some individuals may also use multiple drugs to help alleviate undesired side effects, such as the use of sedatives to overcome the insomnia caused by stimulants. The route of drug administration also impacts drug use. Injected, smoked, and inhaled drugs act more rapidly compared to orally administered drugs, thereby exhibiting a higher reinforcement and addiction potential [10].

Drugs can act through metabotropic and ionotropic mechanisms. Most drugs of abuse act through metabotropic G-protein coupled receptors, which mediate actions in seconds. This process influences the level of monoamine neurotransmitters noradrenaline, dopamine, and serotonin. Dopamine, however, plays a key role in causing euphoria and reinforcing behavior. Other drugs, such as benzodiazepines and phencyclidine, act through the ionotropic mechanism involving ligand-gated ion channels that mediate synaptic transmission in milliseconds. The gamma-aminobutyric acid (GABA) and N-methyl-D-aspartate (NMDA) receptors represent examples of ionotropic receptors [8].

The characterization of antidepressants based on their transporter selectivity is an established practice, which can also be used for the characterization of drugs of abuse [11]. Stimulants can be classified based on their relative serotonergic and dopaminergic activity using the dopamine/serotonin transporter (DAT/SERT) inhibition ratio. Such ratios, derived from *in vitro* studies, help predict the clinical toxicity of new drugs and their addiction potential. A low DAT/SERT ratio (below 0.1) indicates a tenfold greater relative serotonergic versus dopaminergic activity, similar to that for 3,4-methylenedioxymethamphetamine (MDMA). A high ratio (above 10) corresponds to a greater relative dopaminergic activity similar to that for methamphetamine. A high DAT/SERT inhibition ratio indicates strong stimulant effects and a higher potential for addiction [12]. Table 1 provides an overview of different drug classes focusing on drugs—both scheduled and unscheduled—with abuse potential. In addition, the DAT/SERT ratios are presented for stimulants. The following sections in this chapter focus on drug abuse involving conventional drugs, prescription drugs, and NPS. In particular, NPS are discussed in greater detail.

Table 1. Examples of drugs with an abuse potential [12-14].

	<i>Category</i>	<i>Effect</i>	<i>Target of action</i>	
Cannabinoids				
<i>AB-FUBINACA</i> ^A	synthetic cannabinoid	anxiolytic	CB1	
<i>JWH-018</i> ^{A,B}	synthetic cannabinoid	anxiolytic	CB1	
<i>Δ9-tetrahydrocannabinol (THC)</i> ^{A,B}	natural cannabis	anxiolytic	CB1 (partial)	
Hallucinogens				
<i>Diphenidine</i> ^A	phencyclidine analog	dissociative	NMDA	
<i>Lysergic acid diethylamide (LSD)</i> ^{A,B}	ergotamine	psychedelic	DA-SER	
<i>Methoxetamine</i> ^{A,B}	ketamine analog	dissociative	NMDA, DA	
<i>Psilocin</i> ^{A,B}	tryptamine	psychedelic	SER	
Opioids				
<i>Buprenorphine</i> ^B	medium opioid	sedative-analgesic	μ (partial), κ antagonist	
<i>Codeine</i> ^B	weak opioid	sedative-analgesic	μ	
<i>Fentanyl</i> ^B	strong opioid	sedative-analgesic	μ	
<i>Methadone</i> ^B	strong opioid	sedative-analgesic	μ, NMDA	
<i>Tramadol</i>	weak opioid	sedative-analgesic	μ, SER, noradrenergic	
<i>U-47700</i> ^{A,B}	designer opioid	sedative-analgesic	μ	
Sedatives				
<i>Etizolam</i> ^{A,B}	benzodiazepine	sedative-anxiolytic	GABA	
<i>Flubromazepam</i> ^{A,B}	benzodiazepine	sedative-anxiolytic	GABA	
<i>Hydroxyzine</i>	antihistamine	sedative-anxiolytic	H1	
<i>Phenazepam</i> ^{A,B}	benzodiazepine	sedative	GABA	
<i>Pregabalin</i>	gabapentinoid	anticonvulsant-	GABA	
		analgesic-anxiolytic		
<i>Temazepam</i> ^B	benzodiazepine	sedative-hypnotic	GABA	
<i>Zolpidem</i> ^B	imidazopyridine	sedative-hypnotic	GABA	
Stimulants				DAT/SERT^c
<i>Alpha-pyrrolidinovalerophenone (α-PVP)</i> ^{A,B}	cathinone	stimulant	DA	6020
<i>Amphetamine</i> ^{A,B}	phenethylamine	stimulant	DA	40
<i>Cocaine</i> ^{A,B}	cocaine	stimulant	DA-SER	3.1
<i>Desoxypipradrol (2-DPMP)</i> ^{A,B}	pipradrol	stimulant	DA	1328
<i>Ethylone (bk-MDEA)</i> ^A	cathinone	empathogen-stimulant	DA-SER	0.8
<i>Methylenedioxymethamphetamine (MDMA)</i> ^{A,B}	phenethylamine	empathogen	SER	0.08
<i>Methylenedioxypyrovalerone (MDPV)</i> ^{A,B}	cathinone	stimulant	DA	300
<i>Methamphetamine</i> ^{A,B}	phenethylamine	stimulant	DA	22
<i>Methylphenidate</i> ^B	phenethylamine	stimulant	DA	6725
<i>Para-methoxyamphetamine (PMA)</i> ^{A,B}	phenethylamine	empathogen	SER	0.03

^A, Not in normal therapeutic use in Finland; ^B, Controlled under the narcotics law in Finland; ^C, A low DAT/SERT ratio (<0.1) indicates a tenfold greater serotonergic vs dopaminergic activity; a high DAT/SERT ratio (>10) indicates a greater dopaminergic vs serotonergic activity; *CB1*, cannabinoid receptor agonist; *DA*, dopaminergic; *DAT/SERT*, dopamine/serotonin transporter inhibition ratio; *GABA*, gabaergic; *H1*, histamine receptor antagonist; *NMDA*, N-methyl-D-aspartate receptor antagonist; *SER*, serotonergic; *μ*, μ-opioid receptor agonist.

2.1.1 Conventional drugs of abuse

In Finland, the most common conventional drugs of abuse consist of amphetamine and cannabis, while heroin use is virtually non-existent [2,5,15]. In 2012, the number of problem amphetamine users stood at 11,000 to 18,000 [16]. Amphetamines inhibit the transporters of all three monoamine neurotransmitters. They also inhibit the monoamine oxidase and promote the release of monoamines [8]. The enhancement of dopaminergic neurotransmission predominates with amphetamine and methamphetamine, while MDMA primarily increases the levels of serotonin and noradrenaline [12]. MDMA is the prototypical empathogen or entactogen drug producing feelings of emotional empathy while other stimulants arouse and stimulate users [12]. Adverse effects associated with stimulants include tachycardia, hyperthermia, insomnia, anxiety, depression, and paranoia [17]. MDMA abuse is associated with a higher serotonergic toxicity than other stimulants involving risks for hyperthermia, seizures, and cardiac arrest [18].

Cannabis, specifically Δ^9 -tetrahydrocannabinol, acts through cannabinoid receptors CB1 and CB2. The CB1 receptors are primarily situated in the brain, while the CB2 receptors are found in the spleen and the immune system tissues [13]. The psychotropic effects of cannabis are mainly caused by the activation of CB1 receptors [19]. In addition, cannabis appears to enhance dopaminergic activity. The psychotropic effects of cannabis include euphoria, relaxation, and the enhancement of sensory perception. Furthermore, cannabis has analgesic, anticonvulsant, and cardiovascular effects. Adverse effects consist of panic attacks, tachycardia, and depersonalization. Chronic cannabis use at high doses may cause long-term impairment of cognitive abilities [19].

2.1.2 Abused prescription drugs

Contrary to conventional drugs of abuse, prescription drugs typically regarded as medication and endorsed by clinicians give rise to misconceptions whereby they are considered as safe substances of abuse [10]. Often abuse doses, excluding opioids, are much higher than therapeutic doses. In Finland, the abuse of prescription opioids is a major cause for concern. Estimates for the number of problematic Finnish opioid users in 2012 reached 13,000 to 15,000 [16]. The top five drugs causing fatalities in 2013 consisted of four opioids: buprenorphine, tramadol, codeine, and oxycodone [2]. Opioids induce sedative and analgesic effects by responding to opioid receptors μ , κ , and δ , and the opioid receptor-like 1 (ORL1). The μ -receptor is primarily responsible for the analgesic effects of opioids. Strong opioids are used to combat severe pain and in opioid maintenance treatment, while weak opioids typically target mild and moderate pain. Respiratory depression and oversedation represent the most severe adverse effects of opioids. Concomitant use of other central nervous system depressants such as alcohol or benzodiazepines increase the risk of fatal poisoning [20,21].

Pregabalin was the third most common drug causing fatal poisonings in 2013 [2]. Typically, it is used to treat neuropathic pain, epilepsy, and generalized anxiety disorder. Gabapentin, like pregabalin, increases the synthesis of GABA in the brain,

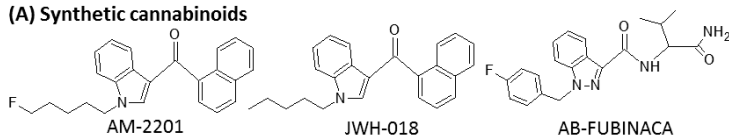
reducing the release of excitatory neurotransmitters. Both of these gabapentinoids—particularly pregabalin—appear to carry an abuse potential [22]. Pregabalin abuse often accompanies multiple substance abuse [22,23] involving higher doses than those used for therapeutic purposes [23]. The adverse effects of pregabalin include dizziness, loss of consciousness, and visual disturbances.

Benzodiazepines are prescribed for anxiety, insomnia, and agitation. In Finland, these widely abused drugs often accompany concomitant prescription opioid abuse [2,20,21]. Benzodiazepines increase the synthesis of the inhibitory neurotransmitter GABA by binding to the GABA-benzodiazepine receptor complex located in the central nervous system. The adverse effects of benzodiazepines include dizziness, lethargy, and rebounding when finishing the use. Chronic use may cause tolerance and dependence.

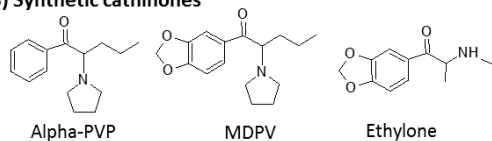
2.1.3 New psychoactive substances (NPS)

In recent years, NPS have gained worldwide popularity as easily accessible and legal, until scheduled, derivatives of classically abused drugs. These novel drugs are often sold through internet sites with intriguing brand names under the guises of herbal incense, research chemicals, bath salts, food supplements, and plant food, and typically carrying the label “not for human consumption” [24-26]. NPS may gain their appeal as drugs of abuse due to the misconception that they are safe alternatives to classic illicit drugs. Furthermore, curiosity arises from their low prices and attractive packaging. NPS are compounds not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, despite potentially posing public health risks. In addition to new molecular compositions, the term “new” comprises substances recently made available on the recreational drugs market regardless of prior concerns regarding their toxicity [25]. Many NPS initially underwent investigation as medicinal drugs, but were rejected due to their harmful side effects or ineffectiveness. Despite preventive actions to schedule NPS as controlled substances, new derivatives with slightly altered structures emerge on the recreational drugs market. Such dynamic changes appear in the number of NPS annually reported by the European Monitoring Centre for Drugs and Drug Addiction and by the United Nations Office on Drugs and Crime. A record 101 NPS were reported through the European Union early warning system in 2014. After having increased for six years, a decrease in the number of NPS was found in 2015, and during 2016, 66 NPS were reported in Europe [5]. The largest classes of emerging NPS in 2016 consisted of synthetic cathinones, synthetic cannabinoid receptor agonists (synthetic cannabinoids), opioids, and substances not fitting in other monitored groups. Figure 1 illustrates the diversity in the chemical structures for select NPS.

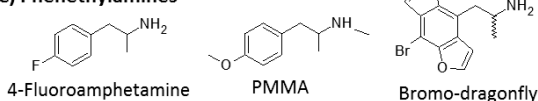
(A) Synthetic cannabinoids



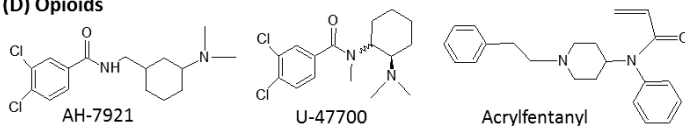
(B) Synthetic cathinones



(C) Phenethylamines



(D) Opioids



(E) Sedatives

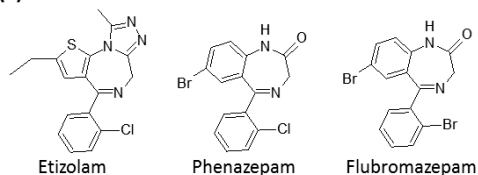


Figure 1. Structures of new psychoactive substances including (A) synthetic cannabinoids, (B) synthetic cathinones, (C) phenethylamines, (D) opioids, and (E) sedatives. *Alpha-PVP*, alpha-pyrrolidinovalerophenone; *MDPV*, methylenedioxypyrovalerone; *PMMA*, para-methoxymethamphetamine.

The desired effects of NPS often resemble those of their controlled counterparts producing feelings of relaxation, euphoria, dissociation, weightlessness, sedation, and anxiolysis [27]. Synthetic cathinones exhibit clinical similarities to amphetamines [26]; correspondingly, these β -ketoamphetamines act as central nervous system stimulants affecting the levels of monoamine neurotransmitters [28,29]. All synthetic cathinones, however, exhibit a higher dopaminergic activity than their non- β -ketoamphetamine analogs suggesting a stronger stimulant effect and a greater risk for dependence [12]. The sympathomimetic effects of both phenethylamines and piperazines are, like those of cathinones, caused by monoamine neurotransmitters [30,31]. The adverse effects of the sympathomimetic drugs include agitation, tachycardia, hypertension, and hyperthermia. The toxidrome in overdoses consists of renal and respiratory failure, psychosis, life-threatening cardiovascular effects, and death [12,13,26,27,30,32].

Similar to natural cannabis, its synthetic derivatives tend towards the cannabinoid receptors CB1 and CB2 [33], but pose a higher risk of overdose [34] due to their more potent receptor agonism. The toxidrome of synthetic cannabinoids is

similar to that of a high dose of natural cannabis, but with more serious adverse effects including extreme agitation, hallucination, hypertension, cognitive impairment, and seizures [27,34-37].

Depressant-type NPS consist of opioids and benzodiazepines that interact with the opioid and GABA receptors, respectively. Both drug classes carry adverse effects resembling those of medicinal opioids and benzodiazepines, although they pose a higher risk for accidental overdose [27]. Data on NPS toxicity—particularly the long-term risks associated with the abuse of these novel drugs—remain scarce. Moreover, with NPS the dose causing the desired effect, impairment and toxicity are fickle given their unpredictable dose-response relationship and often a higher potency than conventional drugs [38].

Given the limited knowledge on the toxicokinetics of emerging NPS, the metabolism of NPS must be studied before establishing a urine drug screening procedure. In particular, researchers must solve whether the parent compounds or metabolites should serve as the main target analytes [39]. The primary phase I biotransformations for NPS include dealkylation, methylation, and hydroxylation, which are catalyzed by cytochrome P450 enzymes. Phase II reactions typically involve conjugation to glucuronic acid via UDP-glucuronosyltransferase enzymes [13]. Contrary to other NPS, synthetic cannabinoids undergo extensive oxidative metabolism, whereby only metabolites are found in the urine [40,41] with a few exceptional cases [42]. Because of the high lipophilicity of synthetic cannabinoids, adipose tissue may serve as an alternative specimen in fatalities given the increased cannabinoid concentrations [43-45].

NPS have caused numerous fatalities [4,15,24,46]. Unlike natural cannabis, deaths either directly or indirectly resulting from synthetic cannabinoids do exist [43-45,47-60]. These reports clearly indicate that synthetic cannabinoids should not be viewed as a safe alternative to marijuana, which poses only a limited acute toxicity. Furthermore, outbreaks of mass poisonings associated with the newer variants of synthetic cannabinoids have been reported [61-65]. The high potency of recent variants, the unknown dose-response ratio, and the poor manufacturing procedures resulting in inconsistent substance compositions in herbal products attribute to such deaths [66]. Moreover, the new practice of selling pure powders of novel cannabinoids may increase the risk of overdose [37].

Current data on treatment strategies for acute NPS intoxication remain limited. Several factors exacerbate the development of such guidelines limiting their usefulness. These include the high frequency of multiple substance abuse, the impurity of NPS products, individual differences in tolerance, and, primarily, the uncertainty of the particular NPS used [67]. In addition to supportive care, antipsychotics and benzodiazepines have been used to manage symptoms, anxiety, and agitation in intoxication caused by synthetic cannabinoids [36,67-70]. Treatment protocols for stimulant toxicity caused by an NPS or for an established recreational drug do not differ greatly. A serotonin antagonist cyproheptadine can directly decrease any effects caused by high serotonin concentrations. In addition, hyperthermia should be treated using cooling measures [70,71]. Sympathomimetic symptoms and agitation associated with piperazine toxicity are managed similar to

stimulants [70], although antipsychotics should be avoided due to the increased probability of extrapyramidal side effects, hypertension, arrhythmias, and thermoregulation interference [72]. Naloxone should always be administered to counter the adverse effects caused by novel opioids [73-75].

While health professionals are likely to come into contact with NPS, most have insufficient knowledge of such substances. Currently, diagnosing NPS use remains difficult within clinical toxicology since the majority of NPS escape routine detection [76]. In general, only specialized drug testing laboratories possess the appropriate instrumentation for NPS detection. A method allowing for the detection of NPS should feature a generic sample preparation and chromatography, and laboratory analysis should cover a wide variety of drugs, both conventional and novel, at the relevant concentration levels. As yet, previously existing methods do not fully meet all of these requirements.

2.2 Drugs of abuse testing

Toxicological drug screening analyses have traditionally relied upon a two-step procedure comprising immunoanalysis and subsequent confirmation of a presumptive positive result using gas chromatography-mass spectrometry (GC-MS). The benefits of atmospheric pressure ionization techniques, such as electrospray ionization (ESI) which often allows the detection of an intact protonated molecule, have shifted the focus to liquid chromatography-mass spectrometry (LC-MS), in particular, LC coupled with tandem mass spectrometry (MS/MS) [7,77-79]. Furthermore, LC-MS allows for the detection of NPS, specifically their polar and less volatile urinary metabolites without requiring laborious derivatization [77,78] while overcoming issues of thermal degradation during GC-MS [80]. In general, data acquisition through MS techniques can fall into targeted and nontargeted approaches. Targeted data acquisition relies on analyte preselection, while nontargeted acquisition enables the collection of data without preset criteria. In particular, the powerful combination of UHPLC and HRMS emerges as an attractive choice for drug screening.

2.2.1 Immunoassays

Immunoassay techniques for drug testing offer simplicity, rapidity, and a high-throughput capacity with minimal sample preparation. Such techniques provide a cost-effective means to detect the presence of a particular drug class, exhibiting an apparent sum concentration above a specific cut-off level. All positive immunoassay results, however, require confirmation using an MS technique if the results are intended to serve a judicial purpose or if sanctions affecting the patient-doctor relationship are likely to follow. The majority of immunoassays only cover classically abused drugs, and consequently, many NPS escape detection [76]. The lack of antibodies hinders the applicability of immunoassays for the detection of NPS. A typical immunoassay drug panel in Finland includes amphetamines,

benzodiazepines, buprenorphine, cannabis, cocaine, methadone, and opiates. Some analytical cross-reactivity using antibodies from established immunochemical screening methods has been reported for a few NPS due to their structural similarities to conventional drugs [76,81-84]. Such cross-reactivity can be regarded as desirable or unwanted depending upon the scope of testing. Regardless, these results appear as false positives (FP) if the confirmation analysis cannot identify the substance causing the positive immunoassay result.

Published immunoassay methods for the urinary metabolites of synthetic cannabinoids relied on enzyme-linked immunosorbent assays (ELISA) [85-88], homogenous enzyme immunoassays (HEIA) [89,90], and biochip array technology-based immunoassay [91]. An acceptable sensitivity and specificity with a cut-off concentration of 5 ng/mL was reached by all assays excluding the biochip array technology-based assay. A higher cut-off resulted in poor accuracy due to the curve of the HEIA response [90], whereas the performance of the biochip array technology improved when the cut-off was increased. The assays targeted the metabolites of JWH-018 [85-87,89,91], JWH-250 [85,91], UR-144 [88], or three different synthetic cannabinoids [90]. The performance of the assays was acceptable with a sensitivity ranging between 80% and 100% and a specificity falling between 82% and 100%. Cross-reactivity was observed towards a few additional cannabinoid variants, while the immunoassay targeting the metabolite of UR-144 showed no cross-reactivity towards other synthetic cannabinoids [88].

Published studies on immunoassays specific for NPS other than synthetic cannabinoids remain limited with several kits for a variety of NPS now commercially available [92]. A method based on ELISA that specifically targets the synthetic cathinones mephedrone and methylenedioxypropylvalerone (MDPV) exhibited experimentally determined cut-off concentrations of 1.25 and 10 ng/mL, respectively. Such low levels indicated that these kits may be suitable for serum samples below overdose concentrations [76]. A better performance was observed for urine samples above the cut-off level proposed by the manufacturer. That is, the increased thresholds for mephedrone and MDPV stood at 7.5 and 40 ng/mL, respectively. This biochip array technology-based immunoassay yielded an extremely high rate of FP leading to a specificity of only 69%, although its sensitivity reached 100%. The instability of the analytes attributed to its weak performance, since samples were stored from two to four weeks at ambient temperature [93]. Another immunochemical application using the biochip array technology showed similar results for designer piperazines based on optimized cut-off levels improving the specificity (91%) with an acceptable sensitivity (94%). Once again, a prolonged sample storage time may have compromised the positivity rate [94]. The biochip technique, however, offered a high-throughput screening for thousands of samples with a short turnaround time [91,93,94].

A major drawback with immunoassays lies in the lack of cross-reactivity of many NPS variants using current antibodies. The continuous variability of the drug scene renders keeping methods abreast of the latest derivatives difficult. Those immunoassays developed specifically for NPS often become outdated once commercially available. NPS screening using immunochemical methods includes

the production, development, and validation steps, often preceded by metabolism studies and the commercial production of new antibodies [87,89,91,93,94]. Such a lengthy procedure makes immunoassays generally less useful for NPS screening, which prefers a method with a greater flexibility, such as HRMS displaying a high sensitivity and specificity [76,90,94].

2.2.2 Pretargeted methods based on LC-MS/MS

LC-MS/MS employing a triple quadrupole or hybrid triple quadrupole linear ion trap MS/MS allows a higher identification power and sensitivity than that obtained by immunoassay. In general, these low-resolution MS (LRMS) approaches require analyte preselection. Not many LRMS-based methods appear in the literature for the simultaneous detection of NPS and conventional drugs [95-97]. A more common approach using LRMS is the development of a method specific to a certain NPS class [42,98-103] or multiple NPS classes [104-110]. However, urine testing methods using an analyte coverage of more than 50 drugs including different classes of NPS remain scarce [95,97,105,111], and all but one [111] were published after the present study (I) was completed. The data acquisition mode for most LRMS methods relied on selected reaction monitoring, excluding a few library search approaches in which the scan mode allowed for the acquisition of product ion spectra [42,97,98,111]. Data were acquired in the positive ionization mode. In two LRMS methods, a separate run was performed for a few analytes in the negative ionization mode [95,103]. Methods for a biological specimen other than urine typically only target the parent compounds, while NPS urinalysis [42,99] should always include at least the primary metabolites. Urine drug screening targeting only parent drugs could, however, be sufficient for stimulant NPS [104,105] although not for synthetic cannabinoids [97].

One disadvantage of NPS screening using LRMS lies in the necessity of knowing the target analyte in advance in order to optimize mass fragmentation. The constant upsurge of novel NPS variants necessitates incorporating them into existing methods [7,112], which is not straightforward using LRMS and requires at least a partial revalidation [42,103,105]. Furthermore, reference standards are needed during method development delaying particularly the detection of NPS metabolites.

2.2.3 Accurate mass measurements

The principle of deducing an empirical formula for a compound using a sufficiently accurate measurement of its ion was introduced as early as the 1950s [113]. Mass accuracy is the deviation between the measured and the theoretical mass of an ion often expressed in parts per million (ppm) [114]. Obviously, the smaller the difference, the better the mass accuracy. Typically, accurate mass measurements with results between 2 to 5 ppm are achieved through periodic calibration [115]. Internal and external calibrations represent strategies for mass correction. Using internal calibration, mass correction is performed using the reference mass peaks in the same mass spectrum as the analyte, while external calibration is performed

using the reference masses from the external mass spectrum acquired using similar conditions as the analyte spectrum [116]. In fact, mass accuracy can be defined as the ability to calibrate the instrument response against a known entity [115]. Acquiring high mass-accuracy data requires high-resolution mass analysis using techniques such as time-of-flight (TOF), orbitrap, magnetic sector, and Fourier-transform ion-cyclotron resonance (FT-ICR) [116]. Mass resolving power expresses the capacity of a mass analyzer to separate ions from an adjacent mass-charge ratio (m/z) demonstrating a measurement precision over a wide m/z range [116,117]. In addition, mass resolution is the measurement of the separation of two adjacent mass spectral peaks. As such, it is defined in two ways depending upon the mass analyzer employed: a 10% valley definition is used for magnetic sector instruments and the full width at half maximum (FWHM) definition for quadrupole, FT-ICR, orbitrap, and TOF. A mass resolving power of 30,000 FWHM and a mass accuracy below 5 ppm are routinely achieved using orthogonal TOF-MS [117]. Table 2 details the terminology involved using HRMS.

Table 2. Key terminology involved in high-resolution mass spectrometry [114,118].

Term	Definition
Accurate mass	The experimentally measured mass of an ion with a known charge.
Exact mass	The calculated mass of an ion with a specified isotopic composition.
Mass accuracy	The difference between the measured (accurate mass) and theoretical value (exact mass) of an ion.
Mass resolution	The measure for separating two adjacent mass spectral peaks, for which the observed m/z is divided by the minimum difference $\Delta(m/z)$ for two ions that can be separated: $(m/z)/\Delta(m/z)$.
<i>10% valley definition</i>	The value for two mass spectral peaks of equal height separated by a valley with a maximum of 10% of the peak height.
<i>FWHM definition</i>	The value for a single mass peak, for which $\Delta(m/z)$ is the full width of the peak at half its maximum (FWHM) height.
Mass resolving power	The capability of a mass spectrometer to provide a defined value for mass resolution.
Monoisotopic mass	The exact mass of an ion calculated using the most abundant isotope for each element.

In mass analysis using orthogonal TOF-MS, the ions are accelerated into the flight tube with an electrostatic field orthogonal to the ion beam axis of the ion source [119]. Ion introduction occurs by ESI enabling the transfer of compounds from the liquid to the gas phase in an ionized state [120]. Next, ions travel a predetermined flight path in a field-free drift space and separate according to their m/z associated flight times [119,120]. Analyte ions often encounter a reflecting electrostatic mirror in the flight path directing them to the detector. A conversion dynode and a channel electron multiplier represent the most common detectors [120]. The combination of TOF and a quadrupole (Q) provides a high sensitivity, a high mass resolution, and a high mass accuracy for both the precursor and product ions. QTOF typically comprises the source inlet, three Q (or a combination of Q and

hexapoles), and the reflecting TOF described above. Q_0 serves as the ion guide, Q_1 represents the mass filter, and Q_2 serves as the collision cell in which ions undergo collision-induced dissociation (CID) with neutral gas molecules (typically nitrogen) to form fragments, namely product ions [121].

HRMS gained interest from various disciplines including proteomics [122], environmental analyses [123], and food toxicology involving pesticides [124] as well as adulterants and contaminants [125]. Today, HRMS is widely used in forensic and clinical toxicology [1,7,78,112], including metabolic studies and both qualitative and quantitative drug screening applications [126]. Furthermore, HRMS remains quite appealing in the analysis of NPS in different biological specimens and was applied to several matrices. Table 3 summarizes HRMS screening methods focusing on conventional drugs of abuse and multiple NPS classes. In the table, only blood and urine testing methods are listed. Several impressive reports documented HRMS methods in the analysis of a specific NPS class [90,127-130] as well as for NPS detection in hair [131-133]. But, these topics lie beyond the scope of this study. The applications listed in Table 3 were primarily performed using QTOFMS, excluding one relying on a single-stage TOF-MS [134] and a few applying the orbitrap technique [135-139]. Across all methods, LC was used with one exception relying on the GC method [140]. A few methods using the negative ionization mode were performed in a separate run [141-145] or in parallel to positive ionization by polarity switching [135,136]. Three methods applied a nontargeted screening approach with no upper limit for analyte coverage [141,142,146]. Analyte coverage ranged from 5 to 40 in those methods focusing on multiple NPS classes, whereas the range for the broader methods reached coverage typically above hundreds of substances. However, prior to the drug screening method described in this thesis (**I**), no similarly comprehensive method for urine testing appeared in the literature, while only two methods were published for blood samples [143,147]. Subsequent to the publication of study (**I**), other HRMS drug testing methods covering both conventional and novel drugs were developed for urine samples [136,142,148].

In most studies, data acquisition involved no preset criteria for the precursor selection. Such data-independent acquisition (DIA) enables the collection of full mass range data above a predetermined intensity threshold. All mass data on both precursor and product ions were collected by alternating the acquisition between low and high collision energies (CE) across a specified mass range. The majority of methods captured fragment data applying a CE ramp, while a single [149] or double [142] CE was also applied. The DIA approach produced nonselective broadband data on mass fragmentation. In three of the DIA approaches, only precursor data were acquired [134,143,145], although two gained access to QTOFMS. Another approach relies on the selection of the precursor in advance of mass fragmentation using data-dependent acquisition (DDA). In DDA, precursor selection was most often based on inclusion lists; yet, one study featured the intensity threshold as the selection criteria [137]. This type of targeted MS/MS approach produces more selective fragmentation data than DIA. Only a few studies have combined approaches involving an inclusion list for the production of selective fragment data and a parallel full-scan MS acquisition. Such a procedure enables data acquisition

for unanticipated precursor ions with [135,148] or without [138,139] fragmentation information. Recently, another approach for QTOFMS data acquisition—named the sequential windowed acquisition of all theoretical mass spectra (SWATH)—was introduced [150]. Similar to DIA, SWATH is also independent of analyte preselection, but acquires mass spectra in smaller m/z windows. Figure 2 illustrates the three approaches to HRMS data acquisition.

Table 3. LC-QTOFMS-based methods for the analysis of conventional drugs and new psychoactive substances (NPS).

Targets	Coverage (n)	Polarity^A	Matrix	Data acquisition
NPS and conventional drugs				
<i>Møllerup et al. 2016 [146]</i>	nontargeted	+	B	DIA
<i>Lung et al. 2016 [141]</i>	nontargeted	+, -	B	DIA
<i>Kinyua et al. 2015 [142]</i>	nontargeted	+, -	U, S	DIA
<i>Helfer et al. 2017 [135]^B</i>	700	+/-	P	DDA + parallel FS (DDA)
<i>Roche et al. 2016 [136]^B</i>	616	+/-	B, S, U	DIA
<i>Telving et al. 2016 [149]</i>	467	+	B	DIA
<i>Roman et al. 2013 [143]</i>	240	+, -	B	DIA
<i>Study (I), 2013</i>	>298 (IV)	+	U	DIA
<i>Pedersen et al. 2013 [147]</i>	256	+	B	DIA
<i>Bidny et al. 2017 [144]</i>	>185	+, -	B	DIA
<i>Teng et al. 2015 [151]</i>	151	+	B	DIA
<i>Guale et al. 2013 [134]^C</i>	>100	+	B, S, U	DIA
<i>Li et al. 2013 [137]^B</i>	65	+	U	DDA
<i>Tsai et al. 2013 [145]</i>	62	+, -	U	DIA, subsequent DDA confirmation
<i>Paul et al. 2014 [148]</i>	62	+	U	DDA + parallel FS (DDA)
<i>Chindarkar et al. 2014 [152]</i>	61	+	U	DIA
Multiple NPS classes				
<i>Concheiro et al. 2015 [139]^B</i>	40	+	U	DDA + parallel FS (prec)
<i>Pasin et al. 2015 [153]</i>	37	+	B	DIA
<i>Montesano et al. 2016 [138]^B</i>	25	+	P	DDA + parallel FS (prec)
<i>Ojanperä et al. 2016 [140]^D</i>	5	+	B	DDA

^A, positive (+) and negative (-) ionization in separate runs (+, -) or by polarity switching (+/-); ^B, orbitrap; ^C, single-stage TOF-MS; ^D, GC; B, blood; DDA, data-dependent acquisition; DIA, data-independent acquisition; FS, full scan; P, plasma; prec, precursor mass data; S, serum; U, urine.

Compound identification was most often based on determining the molecular formula from full-scan data by searching the measured HRMS data against in-house databases of exact monoisotopic masses. A candidate list generated using empirical rules for masses can contain multiple potential formulae, which are further reduced by post-acquisition software algorithms [117]. In addition to RT and precursor ion mass accuracy matching, substance identification often employs product ion mass accuracy and isotopic pattern matches. In addition, the substance identification

power is compromised without using product ions [134,143]. Applying mass accuracy criteria to product ions also reduces FP [148,152,154]. In addition, using predicted data on mass fragmentation [155] and metabolism [156] facilitates the tentative identification of NPS and their metabolites [142,146,148]. Relying on QTOFMS to avoid FN screening results due to detector saturation can be achieved by adding the main isotopes for compounds that occasionally exist at a high concentration into the target database [149].

Another option for compound identification from accurate mass measurement focuses on comparing the measured HR product ion mass spectra to a reference mass spectra [157,158]. Among the methods presented in Table 3, three methods that apply the orbitrap technique [135,138,139] and one using QTOFMS [142] rely on spectrum matching. DDA typically precedes the library search by applying inclusion lists to the masses of interest in order to acquire the selective HR product ion spectra suitable for spectral comparison. In addition to the minimum scores for a library hit, the mass accuracy was exploited by setting the minimum criteria to identify substances.

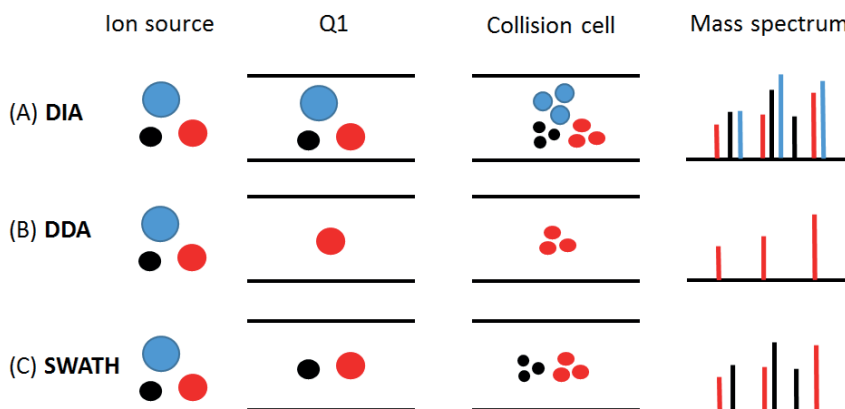


Figure 2. Data-acquisition approaches for high-resolution mass spectrometry: (A) data-independent acquisition (DIA) using the wide quadrupole (Q1) passing mode for precursor ions, (B) data-dependent acquisition (DDA) using a narrow Q1 window, and (C) sequential windowed acquisition of all theoretical mass spectra (SWATH) using the medium Q1 passing mode. Modified from reference [159].

2.3 Epidemiology

Blood-borne infections, overdose morbidity, and mortality constitute some of the most adverse consequences of recreational drug abuse. A large-scale longitudinal study in Finland summarized the characteristics of drug users seeking treatment for substance abuse [3,160,161]. Typically, abuse began with alcohol; during adolescence, the introduction of illicit drugs further evolved to an established drug habit involving intravenous drug use. Among the study population, the primary drugs abused consisted of intravenous opioids, following cannabis and stimulants with a high frequency of multiple substance abuse [3]. Compared to the general population, treatment-seeking drug users had a more than fivefold risk of hospitalization for mental health disorders as the primary diagnosis [160] and a nearly ninefold risk of premature death [161]. Blood-borne infections such as HIV and hepatitis C were more frequent among stimulant users, while those abusing prescription drugs more often received diagnoses of depression and psychosis [160]. The most common causes of death included accidental overdose and suicide [161].

According to the European Monitoring Centre for Drugs and Drug Addiction, the prevalence of cannabis use in Finland currently mimics the European average, whereas the prevalence of amphetamines lies among the highest levels in Europe [5,15]. During 2016, the abuse of methamphetamine, which has historically been restricted to the Czech Republic and Slovakia, significantly increased in eastern Germany and in Finland [5]. Many European countries report heroin abuse as the most prevalent opioid used, while in Finland buprenorphine stands as the most frequently abused opioid. A survey on the prevalence of drug use conducted in 2014 indicated that NPS remain rare in Finland [162]. For instance, the survey revealed that the prevalence of synthetic cathinones and synthetic cannabinoids stood below 1%. Cannabis was the most prevalent drug used (19%) following sedative prescription drugs (5%) and amphetamines (3%). However, a Finnish expert panel study estimated an increase in drug abuse [6]. In particular, they predicted an increase in the abuse of prescription drugs and NPS.

Results from survey-based studies carry several shortcomings. Firstly, people who use drugs may under- or overreport the quantity of drugs they use. Secondly, some populations more poorly participate in surveys including substance abusers. Finally, the drug users are often unaware of the exact drug they are consuming, which is particularly relevant for NPS abuse. An alternative to estimating the patterns and trends of drug abuse employs analytically confirmed results. In Finland, such methodologies used for the evaluation of drug abuse include waste water analysis [163-166], post-mortem toxicology [2,20-22,167,168], studies on driving under the influence of drugs (DUID) [23,169-172], and results from opioid-dependent patients undergoing treatment [173,174]. Although all approaches carry limitations and may not be comparable to the general population, they do provide valuable analytically confirmed information on trends in the Finnish drug use scene.

As such, waste water analyses carried out in 2012, 2014, and 2015 revealed that amphetamines represented the dominant drugs of abuse in Finland [164-166] with a

significant increase in the abuse rate over two years [166]. In terms of NPS, only MDPV and methylone were identified [164,165]. Occasional traces of methylone were found [165], while MDPV was identified in Helsinki [165] and at an exceptionally high rate in the southeastern region of Finland [164,165]. Among weekly trends in drug use, the abuse of MDMA and cocaine were more pronounced during weekends indicating their use as recreational party drugs. By contrast, amphetamine [164-166] and MDPV [164,165] use were associated with problematic drug users due to the consistent concentrations found throughout the week. Similar to urinalysis, the determination of NPS in waste water requires preceding studies on toxicokinetics, as well as analysis of in-sewer stabilities. Moreover, the wide range of NPS together with their sporadic availability on the drugs market hampers detection of any individual substance in a municipal sewage network [175]. Analysis of the ten most frequent NPS in waste water samples collected from eight European cities revealed a low occurrence of NPS with only three synthetic cathinones detected at low concentrations [176]. Therefore, a pooled urinalysis from stand-alone pissoirs (such as those situated in city centers or in close proximity to night clubs) could serve as an alternative to assessing NPS abuse although such results are not comparable to the general population as waste water-based epidemiology [175].

Multiple substance abuse is a common phenomenon among chronic drug users. A study of fatal poisonings among dependent drug users submitted for medico-legal investigation in five Nordic countries in 2012 revealed a high frequency of multiple substance abuse (with 4 to 5 concomitant drugs detected per case) across all countries [167]. In Finland, amphetamine stood as the most common stimulant drug and buprenorphine represented the primary intoxicant in fatal poisonings. Among NPS, few substances were detected across all countries, although the largest repertoire was found in Sweden and Finland. In Finland, the most common NPS were alpha-pyrrolidinovalerophenone (alpha-PVP), MDPV, and paramethoxymethamphetamine (PMMA); in Sweden, ethylphenidate, 5-(2-aminopropyl)indole (5-IT), and the synthetic cannabinoid AM-2201 [167] were detected most often. Post-mortem toxicology in Finland between 2011 and 2013 revealed 80 different NPS in 69 cases, the most frequent being alpha-PVP and MDPV [2]. A synthetic cannabinoid was found in only one case involving multiple substance abuse. In 14 cases, the detected NPS was regarded as the most important contributor to death. Overall, the influence of NPS on the cause of death was insignificant in most cases. Multiple substance abuse involving heavy alcohol use and buprenorphine abuse stood as the most probable cause of death. However, the abuse of stimulant NPS was associated with a higher risk of death by accidental or intentional injury [2]. The entry of a dangerous synthetic opioid U-47700 emerged in 2016, contributing to eight fatalities in Finland [177]. A subsequent national warning was broadcasted to raise awareness among drug users of this hazardous and potent opioid.

A comprehensive post-mortem toxicology database generated in Finland facilitated several epidemiological studies on abused drugs such as prescription opioids [20,21,168] and gabapentinoids [22]. Poisonings by the weak opioids tramadol and codeine were often associated with suicidal overdoses, while the

strong opioids methadone and buprenorphine more commonly caused accidental poisonings [20,21]. Concomitant benzodiazepine and alcohol use often associated with buprenorphine, codeine, and tramadol poisonings increasing opioid toxicity. Although fentanyl was the least-abused opioid, this potent drug caused the highest number of fatal poisonings among known drug users [168]. The typical victim of a fatal poisoning via a prescription opioid was a male drug user aged around 30 years [20,21].

Studies among drivers apprehended for DUID revealed the range of drugs encountered in traffic. Abuse profiles for selected NPS and pregabalin were assessed among apprehended Finnish drivers [23,169-171]. All studied NPS (MDPV, phenazepam, and 2-diphenylmethylpiperidine (2-DPMP)) as well as pregabalin were frequently accompanied by the use of other drugs. Amphetamines and benzodiazepines represented the most common concurrently used drugs. The typical apprehended driver was a multiple substance user and a 30-something man from Southern Finland [169-171]. Phenazepam, however, was often encountered near the Russian border. In comparison to Finland, in Russia phenazepam is used as a prescription drug for anxiety, epilepsy, and alcohol withdrawal syndrome [170]. Moreover, two types of phenazepam users exist: those using multiple sedatives and those self-medicating for stimulant withdrawal. When MDPV first entered the Finnish drugs market, the incidence of MDPV among apprehended drivers was particularly high [169]. However, the prevalence of NPS in traffic and in post-mortem toxicology differs [170,171], possibly resulting from the lower toxicity of some NPS [170]. In both toxicological investigations, the prevalence of MDPV decreased after it was banned [172]. Such a decline in MDPV use appears promising for the public welfare, since MDPV may be associated with a higher risk of suicide [2,172].

Prevalence studies on other clinical samples in Finland include monitoring MDPV use among opioid-dependent patients with incidental amphetamine abuse [173]. Among this narrow study population, MDPV was detected among 26%. Another Finnish study revealed that an inadequate dose of opioid maintenance treatment medication associated with a higher probability of benzodiazepine abuse [174]. These studies emphasize the importance of drug testing within opioid maintenance treatment. In particular, treatment safety may improve if patients with confirmed drug use are denied take-home doses of their substitution therapy [173]. Laboratory-based studies among Finnish drug users remain scarce, although established drug users likely use multiple substances and NPS [4]. Such use calls for new research relying on modern analytical techniques enabling the assessment of accurate drug abuse profiles.

In Sweden, a joint nationwide project STRIDA (an acronym derived from the Swedish name of the project) began in 2010 to monitor the incidence of NPS use by analyzing drug intoxication cases presenting at emergency departments and in intensive care units across the country [178,179]. Project outcomes included a better understanding of clinical features related to NPS intoxication and analytically verified national trends on drug use. The project produced valuable toxicity and prevalence data from NPS intoxication involving novel fentanyl [73,74,180] and

opioid [75] analogs, synthetic cathinones [181-183], synthetic cannabinoids [37], dissociatives [184,185], and other NPS stimulants [186,187]. However, the assessment of a specific toxidrome for a certain NPS was not straightforward due to the high incidence of multiple substance use. A unique ototoxic reaction was, however, observed for the designer opioid MT-45 [75]. Such bilateral hearing loss is typically transient, although one patient showed a permanent defect. Contrary to other NPS studied within the STRIDA project, the abuse of alpha-PVP and MDPV were thought to be particularly common among established stimulant drug users due to their high median age, the high prevalence of hepatitis C, and the high frequency of intravenous administration [181,182]. The drug use situation in Sweden can to some extent reflect the Finnish context given various similarities in drug profiles [167]. As exemplified by the vast number of NPS encountered in the Swedish emergency toxicological setting, such nationwide co-operation facilitates the exchange of information and raises overall awareness of NPS.

3 AIMS OF THE STUDY

This study aimed:

- To develop and validate a multidrug screening procedure enabling the simultaneous detection of both traditional and new psychoactive substances (NPS) by ultra-high performance liquid chromatography/high-resolution quadrupole time-of-flight mass spectrometry (UHPLC-HR-QTOFMS) (**I**).
- To evaluate the capability of the UHPLC-HR-QTOFMS method as a substitute for conventional immunoassays in drug screening (**III**).
- To compare two general mass spectrometry-based workflows for data acquisition and processing (**II**).
- To utilize data-independent broadband mass spectral data followed by a reverse database search for tentative compound identification of NPS metabolites (**I**).
- To demonstrate the applicability of broadband data to identify co-eluting isomeric and isobaric compounds (**II**).
- To apply the developed method to the collection of epidemiological data and to the assessment of patterns of drug abuse among drug users with different treatment statuses (**IV** and **V**).

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals and reagents

The reference standards, which were of pharmaceutical purity, for the majority of drugs were purchased from several pharmaceutical companies. Some NPS consisted of seized material obtained from the Finnish Customs Agency. Reagents for immunoanalysis were provided by Siemens (Erlangen, Germany), Abbott Laboratories (Abbott Park, IL, USA), and Medichem (Steinenbronn, Germany) (**III**). All other chemicals were of analytical or LC-MS-grade purity and were obtained from various suppliers.

4.1.2 Urine samples

Clinical urine samples were collected at health-care units and sent to the laboratory for drug testing (**I**, **II**, **IV**, and **V**), while post-mortem urine samples were collected by forensic pathologists during medico-legal autopsies (**I** and **III**) for routine toxicological screening. In addition, the Institute of Forensic Medicine at the University of Freiburg (Germany) provided urine samples previously confirmed positive for synthetic cannabinoids (**I**). The analysis of some of the submitted samples formed a part of routine clinical treatment for drug users (**IV** and **V**). Drug-free urine samples from healthy volunteers were used for the validation studies (**I** and **II**).

Sample selection was performed using different criteria depending on the study design. To evaluate the applicability of the UHPLC-HR-QTOFMS drug screening method, 50 samples previously found positive for drugs of abuse (**I**) were analyzed. For the performance comparison study, 279 consecutive post-mortem urine samples (**III**) and 50 clinical samples (**II**), including both drug-positive and drug-negative samples, were analyzed using UHPLC-HR-QTOFMS and the results were compared to the immunoassay (**III**) or to DDA data acquisition (**II**), respectively. The results from routine analyses remained blinded until completion of analyses (**II** and **III**). Samples from drug users in treatment were acquired consecutively between October 2013 and April 2014 ($n = 200$, **V**) and from 36 anonymous volunteers collected between December 2013 and March 2014 ($n = 67$, **IV**). A total of 32 anonymous subjects irregularly visiting a harm reduction unit provided 34 samples (**IV**).

All treated study subjects provided their written consent for urine analysis as a part of their treatment (**IV** and **V**). The study protocols were approved by the Medical Director of A-Clinic Foundation (**IV**) and the Institutional Review Board of the Department of Psychiatry, Helsinki University Central Hospital (**V**). The other samples studied used anonymous personal identifiers solely intended for method

development and comparison purposes, and, therefore, were performed with permission from the Department of Forensic Medicine without requiring separate approval from the Ethical Committee (I–III).

4.2 Sample preparation

All urine samples excluding those subjected to immunoanalysis (III) underwent the same sample preparation prior to UHPLC-HR-QTOFMS analysis. Samples were hydrolyzed with β -glucuronidase and extracted using solid-phase extraction. The mixed-mode sorbent comprised reversed-phase (C4) and cation exchange properties. Some modifications were performed during the study: the sample amount was decreased from 1 mL (I) to 0.5 mL (II–V), the volume of reconstitution solvent was decreased from 150 μ L (I) to 75 μ L (III, IV, and V), but increased to 500 μ L (II) when increasing the injection volume from 1 μ L (I, III, IV, and V) to 5 μ L (II). All modifications were made due to the introduction of an upgraded analog to a digital converter resulting in an improved scanning speed, and to widen the dynamic range by avoiding column overload and detector saturation caused by extremely high drug concentrations.

4.3 Instrumentation and analytical methods

4.3.1 Immunoassay

Untreated urine samples were analyzed using a Vitalab Viva analyzer applying the enzyme multiplied immunoassay technique (EMIT) according to the manufacturer's recommendations for the cut-off concentrations (III, Siemens, Erlangen, Germany). Analysis was based on competition between a free drug and an enzyme-labelled drug for antibody binding sites. A sample with a drug concentration exceeding the cut-off produced a measurable absorbance change directly proportional to the urinary drug concentration.

4.3.2 Ultra-high performance liquid chromatography/high-resolution quadrupole time-of-flight mass spectrometry (UHPLC-HR-QTOFMS)

The UHPLC-HR-QTOFMS system consisted of a maXis Impact (I and III) or an Impact HD (II, IV, and V) mass spectrometer (Bruker Daltonics, Bremen, Germany) combined with a Dionex Ultimate 3000 series UHPLC (Sunnyvale, CA, USA). The UHPLC instrument included a vacuum degasser, a binary pump, a temperature-controlled autosampler, and a column oven. Chromatographic separation was performed using a Waters HSS T3 (150 mm \times 2.1 mm, 1.8 μ m) column and an equivalent precolumn (2.1 mm \times 5.0 mm) in the gradient mode at

60°C. The mobile phase consisted of 2-mM ammonium acetate in 0.1% formic acid and methanol. The flow rate was 0.3 mL/min.

The HR-QTOFMS instrument was equipped with an ESI source and a six-port divert valve. The mass resolving power specification of the instrument for m/z 1222 was $\geq 40,000$ FWHM. In practice, the median mass resolving power for alpha-PVP (m/z 232.1696) was 24,700 FWHM. The instrument was operated in the positive ion mode acquiring data at an m/z range of 50 to 700 using DIA (I–V) and DDA (II). Mass fragmentation was performed with broadband collision-induced dissociation (bbCID, I–V) or with a preselected CE for targeted compounds (II). Both external instrument calibration and post-run internal mass scale calibration of individual samples were performed with a sodium formate solution using nine cluster ions with exact masses between 90.9766 and 634.8760. Figure 3 presents the workflow for urine drug screening employing DIA and DDA.

The data processing software for the UHPLC-HR-QTOFMS acquisition data consisted of DataAnalysis (I: 4.1 (version 358); II–V: 4.2 (version 376)), Target Analysis 1.3 (I–V), and LibraryEditor 4.1 (II, Bruker Daltonics). Substance identification was based on an automated post-acquisition reverse database search with preset reporting criteria (I–V) and a comparison of the measured product ion spectra to the reference mass spectra (II). For the database search, drug identification criteria were set for the mass accuracy (I: ± 3 mDa; II–V: ± 2.5 mDa), peak area counts for the product (I: 100; II–V: 2,200) and precursor ions (I: 10,000; II–V: 20,000), and RT (I–V: ± 0.2 min). The value for the precursor isotopic pattern match served as an additional attribute for identification. In addition, individual area criteria were applied for synthetic cannabinoids, internal standards, and for a few individual drugs (II–V). Compounds fulfilling the predetermined criteria were reported in the list of positive findings. For the library search (II), the measured and the reference spectra were compared using scores for spectral similarity.

An in-house database of exact masses (I–V) and a spectrum library of 200 compounds (II) were created by analyzing the reference standards of drugs, and further assigning the molecular formulae for the precursor and product ions and the most characteristic product ion spectra (II). Initially, the database of toxicologically relevant drugs of abuse comprised 277 compound entries (I). The analyte panel was further extended to cover more drugs and emerging NPS together with their known or predicted metabolites yielding a collection of approximately 550 compound entries (III, IV, and V). For the acquisition mode comparison, a subset of the database containing 200 compounds was created to match the scope of the spectrum library (II). The ACD/MS Fragmenter (version 11.01, Advanced Chemistry Development, Toronto, Canada) and SmartFormula3D (Bruker Daltonics) software programs were used to assign molecular formulae for product ions when adding new entries to the database.

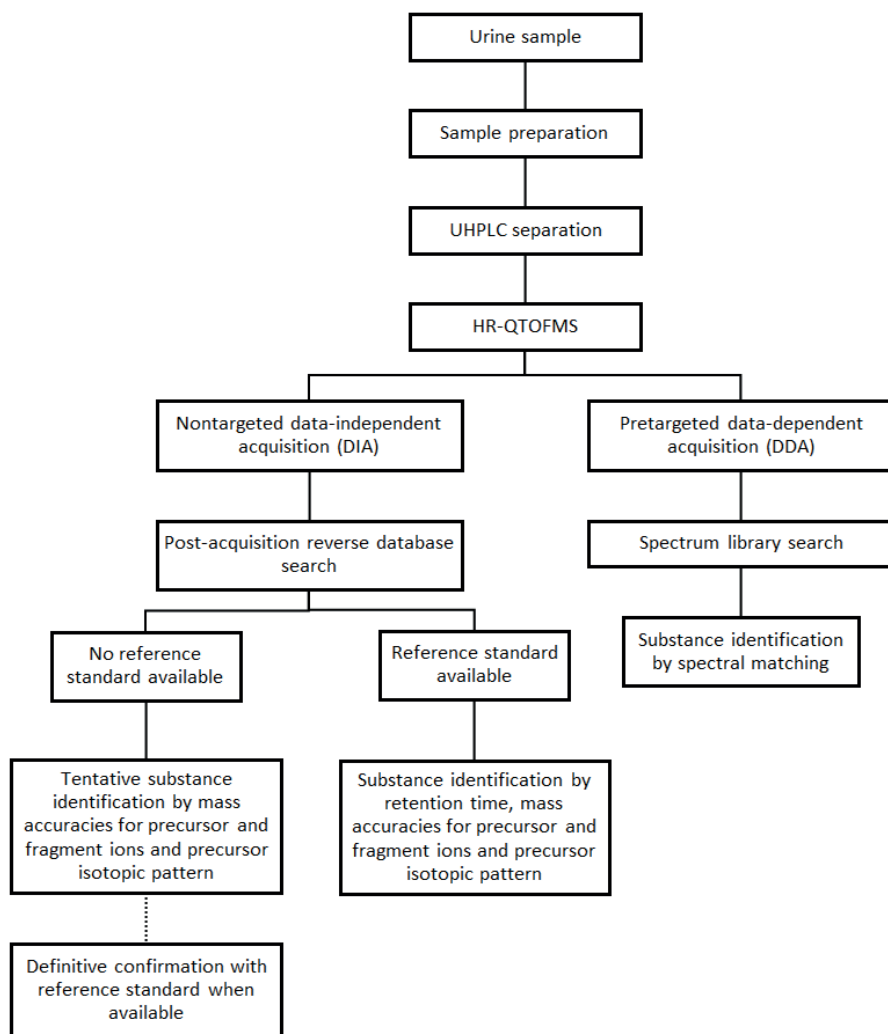


Figure 3. Workflows for post-targeted (I–V) and pretargeted (II) urine drug screening approaches using UHPLC-HR-QTOFMS.

5 RESULTS AND DISCUSSION

5.1 Drug screening based on data-independent acquisition (DIA)

A urine screening method based on UHPLC coupled with HR-QTOFMS was developed for drugs of abuse featuring a scope beyond ordinary drug testing. Using broadband DIA, both conventional drugs and NPS, including the urinary metabolites of synthetic cannabinoids, were encompassed for the first time. In previous methods, no more than nine metabolites for synthetic cannabinoids were included [42,99,127]. More recently, two HRMS-based screening methods relying on DIA were developed exclusively for the urinary metabolites of synthetic cannabinoids [90,129]. Moreover, an HRMS screening method covering hundreds of drugs including synthetic cannabinoids was developed for blood samples [149] as well as a single-stage TOF-MS screening method involving only the parent synthetic cannabinoids for blood, serum, and urine samples [134]. However, the comprehensive screening method developed in this thesis (**I**) remains quite unique in its ability to cover both the urinary metabolites of synthetic cannabinoids and other drugs of abuse including amphetamines, cathinones, cocaine, hallucinogens, natural cannabis, opioids, and sedatives. One reason this challenge persists is the implementation of lipophilic synthetic cannabinoids and hydrophilic stimulant NPS into the same method, while maintaining a satisfactory analysis time. A different solution lies in applying two separate chromatographic runs with a higher proportion of organic content in the mobile phase for synthetic cannabinoids, as illustrated through the use of an LC-MS/MS-based screening method for hair samples [108].

Initially, the in-house database included 277 compound entries involving exact masses for the precursor ions and for the majority of product ions, in addition to RT when a reference standard was available (**I**). The database was continually updated with emerging NPS (**III**, **IV**, and **V**) to contain approximately 550 compound entries of toxicological relevance. Incorporating rarely occurring substances, such as psilocin and lysergic acid diethylamine (LSD), into the screening method fulfils the requirement for systematic toxicological analysis [7]. Substance identification was completed through post-acquisition reverse database search using the acceptance criteria for mass accuracy, area counts for both product and precursor ions, and RT. The precursor isotopic pattern supported the identification.

Table 4. Limit of identification (LOI), mass accuracy, and mass resolving power for selected compounds (I).

		Mass accuracy (mDa)		Mass resolving power (FWHM)	
Compound	LOI (ng/mL)	A	B	A	B
Cannabinoids					
AM-2201	0.4	0.6	0.4	30,577	30,158
HU-210	10	0.1	0.4	24,120	17,333
JWH-018-5-pentanoic acid	2.0	0.1	0.2	25,957	23,689
THC-COOH	15	0.2	0.3	19,764	18,212
Cathinones					
MDPV	0.8	0.2	0.2	27,936	25,417
Methylone	1.5	0.1	0.1	20,445	18,759
Opioids					
Buprenorphine	1.0	1.3	1.1	27,116	25,417
Morphine	2.0	0.2	0.1	19,783	18,016
Amphetamines					
MDMA	0.9	NA	NA	NA	NA
Methamphetamine	6.5	0.4	0.5	16,959	16,324

A studied in a neat standard, LOI and B in urine samples spiked before extraction. FWHM, full width at half maximum; MDMA, methylenedioxymethamphetamine; MDPV, methylenedioxypropylvalerone; NA, not assigned; THC-COOH, 11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol.

The sensitivity of the method was adequate for the screening of conventional drugs and NPS as shown in Table 4, which provides LOI for selected drugs of abuse. LOI was defined as the concentration at which the compound was identified using the preset acceptance criteria. In practice, this meant a precursor area roughly twofold the minimum acceptance criterion. The median LOI for the representative set of 75 drugs of abuse reached 1 ng/mL. Cannabinoids lacking nitrogen possessed the highest LOI, which were poorly ionized in the positive mode making their incorporation into multi-analyte methods challenging [42]. For such compounds, a separate analysis in the negative ionization mode could allow for a better ionization efficiency [95,145]. Another option might rely on employing an instrument capable of rapid polarity switching to achieve a better LOI [135].

For a majority of the 20 model compounds, the matrix effect, recovery of extraction, and overall process efficiency were adequate within the observed ranges of 46% to 129%, 27% to 82%, and 15% to 81%, respectively. The high lipophilicity among synthetic cannabinoids was regarded as a crucial attribute for their poor recovery given their incomplete dissolution in the reconstitution solvent containing 45% methanol. In addition, other researchers reported a poor recovery for synthetic cannabinoids in multi-analyte drug testing, concluding that achieving consistently high recoveries is difficult among compounds with diverse physicochemical properties [134]. A further increase in the methanolic content poorly affected the chromatographic peak shape of early eluting polar compounds. However, neither

the mass accuracy nor the mass resolving power was significantly affected by the addition of the urine matrix (Table 4). No instability for analytes kept in the autosampler for 13.5 h was observed, excluding two synthetic cannabinoids with a possible downward trend in their concentration. Thus, the method appears feasible for routine casework, since the screening results were in good agreement with confirmation analyses. Moreover, analysis of urine samples previously found positive for the synthetic cannabinoids JWH-018 and AM-2201 using an LC-MS/MS method [127] revealed a new metabolite, JWH-072-propanoic acid, first described by Lovett et al. [188]. Figure 4 illustrates the tentative identification of this major urinary metabolite of JWH-018, JWH-073, and AM-2201. In the MS and broadband MS spectra across the chromatographic peak of JWH-072-propanoic acid, the accurate masses of the precursor and product ions were found with a sufficient mass accuracy enabling the tentative identification of the synthetic cannabinoid metabolite.

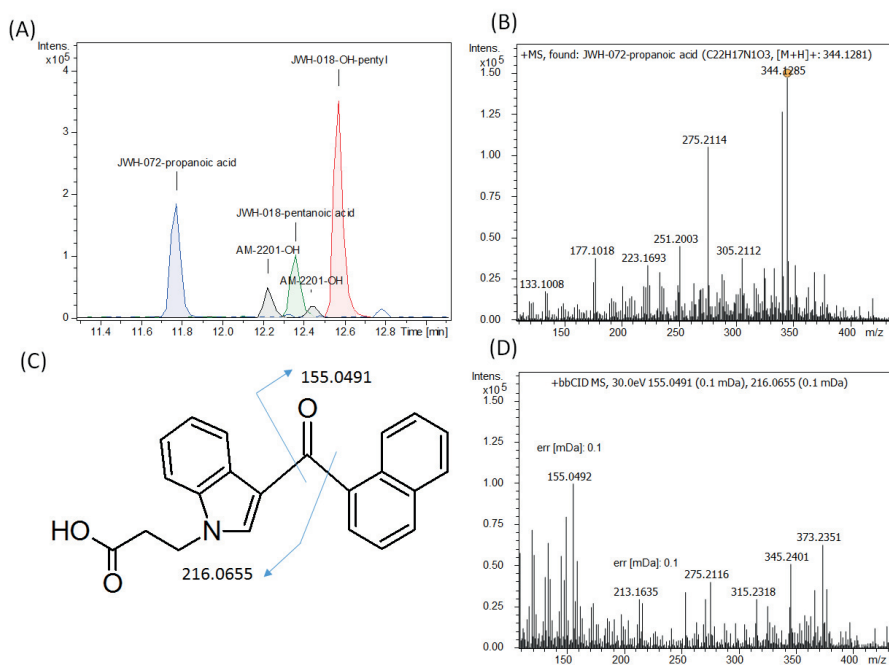


Figure 4. Tentative identification of JWH-072 propanoic acid in a urine sample (I). (A) Extracted ion chromatograms of the metabolites of synthetic cannabinoids, (B) nonselective MS full mass range spectrum, and (D) the broadband spectrum at the corresponding retention time for JWH-072-propanoic acid. (C) The arrows represent the postulated fragmentation sites.

Automated post-acquisition data processing and reporting involved internal mass scale calibration for each sample, the generation and integration of extracted ion chromatograms followed by the retrieval of an average mass spectrum across the peak, a reverse database search with predefined tolerances for the mass and RT, and generating and printing a legible report of positive findings. Figure 5 provides an

example of a truncated urine sample results report. The software generates a user-friendly report including the compound name, molecular formula, RT, and mass deviations from the reference values, isotopic pattern match (mSigma), precursor area, ion intensities (not shown), and mass resolving power. The colored lines indicate those compounds that fulfilled the predefined acceptance criteria. The first column lists the scores for the identification ranging from one to four. The presence of product ions (Q1 and Q2) and small deviations for the RT, mass, and mSigma resulted in a maximum score for an amphetamine. By contrast, the poor identification score for an alpha-PVP metabolite resulted from a lack of the corresponding reference standard.

The time required for automated post-acquisition data processing and reporting was less than one minute per sample, followed by a few minutes to interpret the results report. The entire interpretation process involved a minimal degree of manual review. Occasionally, manual inspection of extracted ion chromatograms was necessary to rule out low-abundant positive findings caused by interfering matrix (III). In another DIA drug screening approach using an exact mass database search, a comparable amount of time was required for data interpretation, given that 48 blood samples took from 30 to 60 minutes to interpret [147]. However, in a DDA-based drug screening procedure, the post-run analysis involving database and library identification, manual exclusion of FP findings, and the report generation required 10 to 30 minutes per chromatogram [157]. A broadband DIA drug screening procedure, combining a post-targeted database search with a nontargeted workflow, required 5 to 30 minutes per blood sample [146]. The time requirement was estimated for an experienced analyst and depended on the number of tentative identifications.

Found	Compound Name	Reg No	Mol Formula	d RT [min]	Err [ppm]	Err [mDa]	mSigma	Area	RT exp [min]	m/z calc	Q1 1 m/z exp	errmDaQ1	Q1 101 2 m/z exp	errmDaQ1	Q1 2 errmDa	Resolution
4	AMPHETAMINE	121	C9H13N1	-0.08	-1.9	-0.3	5.1	3917577	3.91	136.1121	91.0542	0.1	119.0855	0.1	20543	
4	P-OH-AMPHETAMINE	122	C9H13NO1	0.06	-0.6	-0.1	17.8	583149	1.73	152.1070	107.0491	0.2			17306	
3	BUPRENORPHINE	231	C29H41NO4	-0.06	-2.1	-1.0	16.5	4309517	9.35	408.3108					34002	
3	NORBUPRENORPHINE	232	C28H39NO4	-0.09	-1.6	-0.7	18.0	1322375	7.97	414.2639					24649	
2	NALOXONE	233	C19H21NO4	-0.10	-1.8	-0.6	41.1	35229	2.18	328.1543	310.1438	1.3	253.1097	-0.9	17429	
3	MORPHINE	333	C17H19NO3	0.09	0.3	0.1	3.5	211443	1.43	296.1438					18275	
3	MORPHINE	333	C17H19NO3	-0.05	-0.1	0.0	8.3	530897	1.43	296.1438					19128	
4	NORDIAZEPAM	442	C15H11ClN2O1	-0.07	-0.7	-0.2	23.5	468695	11.53	271.0633	140.0262	0.2	243.0684	-0.4	19478	
4	TEMAZEPAM	443	C16H13ClN2O2	-0.02	-1.6	-0.5	27.0	1277258	11.25	301.0738	255.0684	0.4			23519	
4	OXAZEPAM	444	C15H11ClN2O2	-0.04	-0.9	-0.3	12.4	625170	10.79	287.0582	241.0527	0.1	231.0684	-0.2	20726	
4	ALPHA-PVP	531	C15H21NO1	-0.05	-0.9	-0.2	7.7	40764952	6.59	232.1696	126.1277	0.5	185.0335	0.4	32659	
4	ALPHA-PVP METAB 1	532	C15H23NO1	0.05	-0.9	-0.2	6.6	48900132	6.93	234.1852	216.1747	0.4	173.1199	0.3	32897	
2	ALPHA-PVP METAB 1	532	C15H23NO1	-0.24	-1.4	-0.3	12.4	2262313	6.93	234.1852	216.1747	0.0	173.1199	0.1	23435	
1	ALPHA-PVP METAB 2	533	C15H21NO2	-4.16	0.8	0.2	76.8	584893	0.00	248.1645					19480	
1	ALPHA-PVP METAB 2	533	C15H21NO2	-6.30	-0.8	-0.2	6.4	865921	0.00	248.1645					20500	
1	ALPHA-PVP METAB 2	533	C15H21NO2	-9.99	-1.4	-0.3	11.9	5549473	0.00	248.1645					29684	
1	ALPHA-PVP METAB 2	533	C15H21NO2	-10.69	-1.3	-0.3	12.5	16442107	0.00	248.1645					32804	
3	DIETHYLETHYRAMADOL	662	C15H23NO2	-0.09	-1.4	0.4	16.9	92124	4.99	250.1802					17838	
2	NORTRAMADOL	663	C15H23NO2	0.16	0.6	0.2	10.6	188308	6.61	250.1802					17864	
3	NORTRAMADOL	663	C15H23NO2	0.00	-1.2	-0.3	11.8	807654	6.61	250.1802					20498	
2	COCAINE-D3	99999	C18H18D3NO3	-0.02	3.7	1.1	32.7	104326	2.40	303.1783					16784	
2	ETHYLONE	99999	C12H16NO3	-0.19	0.0	0.0	6.6	217318	3.87	222.1125	174.0913	-0.1			18277	
3	MDMA(Ecstasy) D5	99999	C11H10D5NO2	-0.06	0.1	0.0	3.8	584587	4.10	199.1489					18258	
2	PV8	99999	C17H25NO1	-0.11	-0.6	-0.2	6.5	65365392	8.87	260.2009	154.159	0.4	91.0542	0.2	28177	
3	THCA-D3	99999	C21H26D3O4	-0.09	1.9	0.7	21.3	98639	14.36	348.2249					10564	
4	JWH-G18-D9	99999	C24H14D9NO1	-0.10	-0.5	-0.2	6.1	384364	14.71	351.2417	155.0491	-0.1	223.1791	-0.9	19578	

Figure 5. Truncated results report using the reverse database search with automated reporting and printing of positive drug findings from a drug user's urine sample (II). Compounds with identification scores of four and three (green lines) represent compounds fulfilling the acceptance criteria with a minimum deviation for the mass (± 1 mDa) and retention time (RT, ± 0.1 min). Compounds with a score of two (yellow lines) indicated a greater but acceptable deviation for the mass (± 2.5 mDa) or RT (± 0.2 min). Compounds scoring one (white lines) indicated that the identification is based on an accurate mass only.

5.2 Performance comparison

The performance of UHPLC-HR-QTOFMS screening employing broadband DIA was compared to an immunoassay (III) and to the DDA mode applying the same instrument setup (II). This technique comparison was carried out in terms of the scope, flexibility, sensitivity, and reliability of identification based on the analysis of 279 post-mortem (III) or 50 clinical (II) urine samples.

5.2.1 Data-independent acquisition (DIA) vs immunoassay

Based on a comparison using established confirmation analyses, the screening results by immunoassay and broadband DIA were considered true positive (TP), false positive (FP), true negative (TN), or false negative (FN), from which the sensitivity (1) and specificity (2) were calculated using the following equations:

$$(1) \quad \text{Sensitivity (\%)} = (TP / (TP + FN)) \times 100 \text{ and}$$

$$(2) \quad \text{Specificity (\%)} = (TN / (TN + FP)) \times 100.$$

Table 5 shows the sensitivity and specificity values for those drugs included in the immunoassay panel. FN results from the immunoassay were primarily due to the method's high cut-off limits. Amphetamines and opioids possessed the highest limits and, consequently, the lowest sensitivity values. FP results from the immunoassay were likely due to interfering matrix components. The highest number of FP were obtained for buprenorphine, resulting in the lowest specificity. Adjusting cut-off levels to lower concentrations than normally used would improve the sensitivity, but compromise the specificity [189]. By comparison, UHPLC-HR-QTOFMS yielded one benzodiazepine FN due to detector saturation. This analysis was performed using the maxis Impact mass spectrometer, but the current Impact HD with an improved analog-to-digital converter would likely enable correct identification due to its wider dynamic range. Moreover, there were five FP using UHPLC-HR-QTOFMS for codeine and norcodeine, thus slightly diminishing the method's specificity for opioids. In all of these cases, supposedly a cleavage of water (spontaneous fragmentation within the ion source) from the simultaneously occurring oxycodone metabolites gave rise to structures corresponding to these opioids. UHPLC-HR-QTOFMS revealed some additional drug findings of high toxicological relevance beyond the immunoassay drug panel. Such additional confirmed findings included both prescription drugs and NPS.

Several previous studies have suggested that LC-MS/MS could serve as an alternative to immunoassay [98,104,110]. In accordance with the results here, QTOFMS in particular appears more suitable for drug screening than immunoassay [90,145,147]. To date, however, no equally comprehensive evaluation substituting immunoassay using QTOFMS for urine drug screening exists beyond the present study (III). One study claimed the superiority of single-stage TOF-MS urine drug screening over immunoassay based on 800 authentic samples, but a lack of diagnostic product ions weakened its identification power [190].

Table 5. Cut-off concentrations and sensitivity and specificity values for UHPLC-HR-QTOFMS and immunoassay screening (III).

	<i>UHPLC-HR-QTOFMS</i>			<i>Immunoassay</i>		
	<i>Cut-off</i> <i>(ng/mL)</i>	<i>Sensitivity</i> <i>(%)</i>	<i>Specificity</i> <i>(%)</i>	<i>Cut-off</i> <i>(ng/mL)</i>	<i>Sensitivity</i> <i>(%)</i>	<i>Specificity</i> <i>(%)</i>
Amphetamines	6.5 ^A	100.0	100.0	300.0	64.0	98.0
Buprenorphine	1.0	100.0	100.0	1.0	89.0	96.0
Benzodiazepines	15.0 ^B	99.0	100.0	200.0	78.0	100.0
Cannabis	15.0 ^C	100.0	100.0	20.0	79.0	100.0
Opioids	2.0 ^D	100.0	98.0 ^E	300.0	71.0	97.0

Assigned for: ^A, methamphetamine; ^B, oxazepam; ^C, 11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol; ^D, morphine; ^E, codeine.

5.2.2 Data-independent acquisition (DIA) vs data-dependent acquisition (DDA)

In addition to nonselective broadband DIA with no quadrupole selectivity, another QTOFMS approach relies on collecting selective MS/MS data by DDA using preset criteria for the selection of precursor ions. A scheduled precursor list of 200 compounds was used to trigger CID with compound-specific CE. While substance identification in DIA relied on a post-targeted reverse database search, the DDA method employed an in-house spectrum library search yielding scores for similarities between measured and reference mass spectra. Both the database employed in the broadband DIA mode and the spectrum library in the DDA mode comprised the same selection of 200 drugs. The performance evaluation of the acquisition modes for drug testing was performed on 20 model compounds using a single HR-QTOFMS instrument setup examining the sensitivity, specificity, spectral repeatability, and applicability to the casework.

The selection of an appropriate acquisition mode depends on the purpose of the drug screening application. When investigating NPS use, prior knowledge is often missing. Therefore, approaches based on nontargeted data acquisition that produce the full mass range data on precursor and product ions are preferred. Yet, with pretargeted methods that enable quadrupole selectivity, qualitatively better product ion spectra are obtained. Studies that compare acquisition modes using a single instrument remain scarce. Applying the orbitrap technique, three acquisition approaches were compared for pesticide residue analysis: DDA, broadband DIA,

and a variable DIA that acquires mass data in segments similar to the SWATH technique [191]. Employing QTOFMS, two studies compared the toxicological screening performance between SWATH and DDA [192,193]. In drug discovery and development, SWATH, broadband DIA, and DDA were compared using a single QTOFMS-instrument [150,159]. However, prior to the present study (II), no comparison exists examining broadband DIA to a scheduled precursor list-type DDA drug screening technique using a single QTOFMS instrument setup.

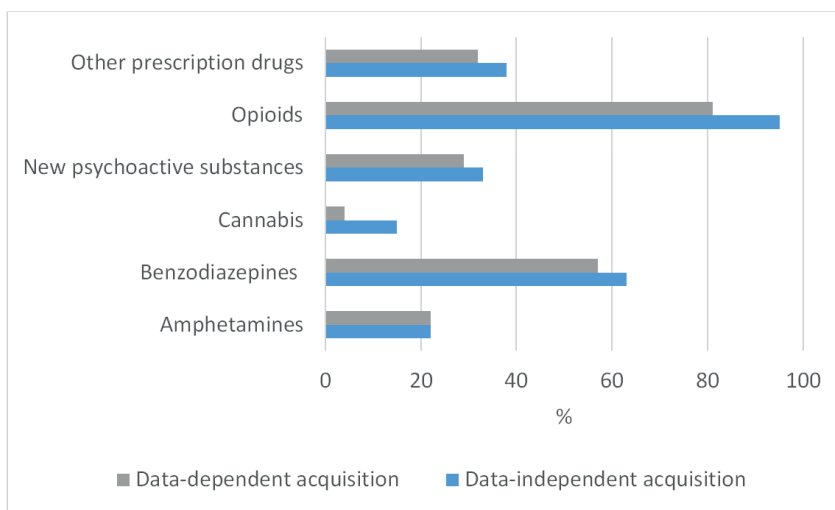


Figure 6. Number of drug identifications in 50 authentic urine samples using UHPLC-HR-QTOFMS analyzed by data-independent (DIA) and data-dependent acquisition (DDA) modes for six drug groups (II).

Among 20 model drugs, 13 yielded a better LOI using DIA than DDA. Using DIA, an acceptable LOI was obtained for a majority of drugs, whereas using DDA a particularly high LOI (≥ 64 ng/mL) was observed for quetiapine, some benzodiazepines, and cannabis. Moreover, DIA proved superior in discriminating co-eluting isomeric compounds. Similar to other studies [192,193], DIA, comprising a wider range of different compounds, produced a higher number of drug identifications in authentic urine samples compared to DDA (Figure 6). Although DIA revealed a higher number of positive cases, DDA performed acceptably in screening for amphetamines, benzodiazepines, NPS, and prescription drugs. However, DDA appears unsuitable for screening for cannabis and opioids, and, in particular, buprenorphine.

The reverse database search using DIA tolerated interferences caused by matrix and co-eluting compounds, while the spectral comparison of product ion spectra acquired using DDA appeared to depend more on spectrum purity. The library search relied on both a forward and reverse search. Applying only the reverse search protocol could work using DDA, since additional peaks from matrix and co-eluting compounds would be ignored in most cases when matching measured and library spectra. Figure 7 illustrates the identification of a synthetic cannabinoid (AB-

FUBINACA) metabolite using both DIA and DDA. Although DDA yields cleaner spectra with appropriate fragmentation patterns, fewer matrix interferences, and excellent scores for the spectral match, substance identification using broadband DIA remains efficient despite the mixed spectra. The overall performance of post-targeted broadband DIA drug screening appeared superior to pretargeted DDA enabling better sensitivity and applicability to casework, along with the flexibility required for the incorporation of emerging NPS. However, subsequently acquired DDA product ion spectra can be useful for occasional confirmation purposes.

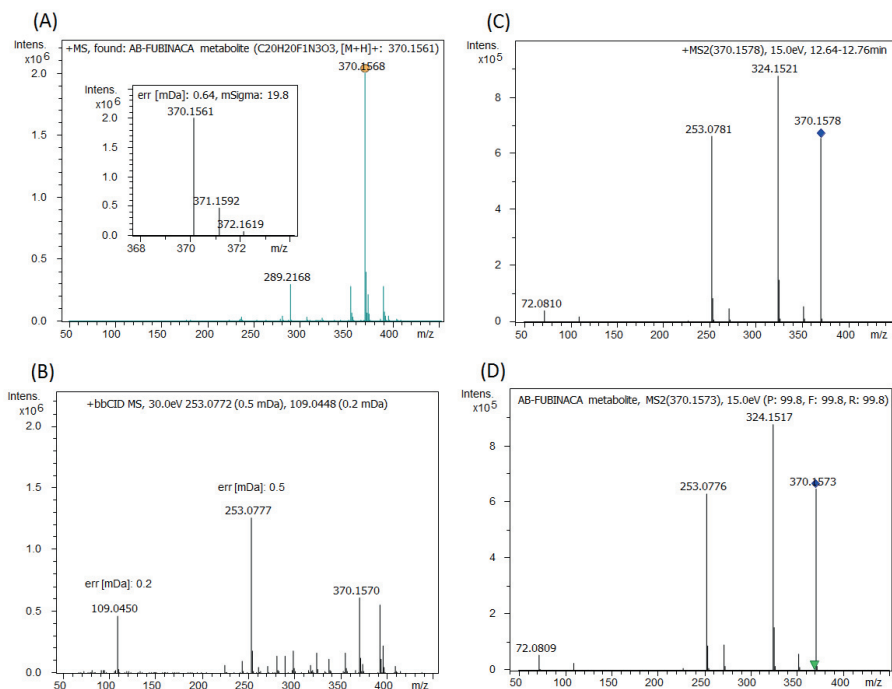


Figure 7. Identification of the AB-FUBINACA metabolite in urine using (A–B) data-independent acquisition (DIA) followed by a reverse database search and using (C–D) data-dependent acquisition (DDA) followed by a spectrum library search (II). (A) Nonselective MS full mass range spectrum with a simulated theoretical spectrum for the precursor, (B) broadband sum spectrum with accurate masses for product ions at a corresponding retention time, (C) selective MS/MS spectrum measured with a preset collision energy, and (D) a library match with scores (%) for purity (P), forward fit (F), and reverse fit (R).

The MS-related definitions used throughout this thesis conformed to the recommendations by the International Union of Pure and Applied Chemistry (IUPAC) [118]. However, the definitions for forward and reverse library searches often rely on opposing meanings. One should always specify which definition is used when comparing procedures for a library search and the corresponding library scores for the spectral match.

5.3 Drug abuse patterns among drug users

In Finland in 2015, the top three drugs causing drug abusers to seek treatment consisted of opioids, stimulants, and cannabis [194]. Estimates suggest that only 20% of problem opioid users attended medicinal opioid maintenance treatment in 2012 [16]. Patients undergoing treatment in 2011 received a buprenorphine-naloxone co-formulation (58%), methadone (38%), or buprenorphine alone (4%) for opioid dependence [195]. In a clinical toxicology setting, the commonly employed immunoassays remain incapable of detecting accurate drug use profiles, only providing drug class-specific results. Drug testing using comprehensive UHPLC-HR-QTOFMS significantly contributes to the clinical assessment and treatment of drug users by providing toxicology results beyond the typical drug testing repertoire.

The UHPLC-HR-QTOFMS screening was used to analyze samples acquired from two rehabilitation clinics supported either by a non-governmental organization (**IV**) or by a municipality (**V**), and from a harm reduction unit aimed at providing counseling for problematic drug users (**IV**). Samples were taken from subjects undergoing (**V**) or in queue for opioid maintenance treatment (**IV** and **V**), in drug withdrawal treatment (**IV**), suspected of acute intoxication (**IV**), and from untreated drug users with self-reported NPS abuse who irregularly visit a harm reduction unit (**IV**). The sample population in study (**IV**) primarily comprised drug abusers undergoing withdrawal treatment, while only a few received opioid maintenance treatment. Subjects receiving treatment visited rehabilitation clinics routinely, while anonymous subjects not attending drug dependence treatment merely received counseling on matters related to social services and health care from harm reduction unit.

Subjects utilizing services provided by a non-governmental organization (**IV**) consisted of two groups: drug users attending treatment for drug dependence and subjects visiting a harm reduction unit receiving services other than medically supervised treatment. For the assessment of drug abuse the prescribed medicines reported by the subject or by the treating physician were excluded. In total, 79% of all analyzed samples were positive for abused drugs. Subjects not receiving treatment exhibited higher drug abuse rates than those receiving treatment (Figure 8). However, similar rates of buprenorphine and benzodiazepine abuse appeared among both groups. Multiple substance abuse occurred in both groups of drug users, but was more pronounced among those not receiving treatment. In addition, the abuse of the prescription drugs pregabalin, gabapentin, and methadone always associated with other concurrently abused drugs. Likewise, NPS always appeared with other abused drugs. The number of concurrently abused substances by drug users attending and not attending treatment reached 2.1 and 3.9, respectively. The corresponding number for those with analytically confirmed NPS abuse was particularly high at 4.8 drugs. As illustrated in Figure 8, the typical pattern of drug abuse among those receiving treatment consisted of buprenorphine, benzodiazepine, and occasionally amphetamines. The abuse pattern among those not receiving treatment often included buprenorphine, amphetamine, cannabis, benzodiazepine, and alpha-PVP.

All subjects attending municipal opioid maintenance treatment (V) received either methadone (72%) or a buprenorphine-naloxone co-formulation (28%) as substitution for opioid dependence. A total of 46% of samples were positive for abused drugs, while over half of the samples tested positive for more than one abused drug. Among the positive samples, 26% showed signs of the simultaneous abuse of two drugs and 34% indicated that more than two drugs were simultaneously abused. Similar to subjects receiving treatment in study (IV), the mean number of concurrently abused drugs reached 2.1. Benzodiazepines represented the most commonly abused drug group, followed by amphetamines, cannabis, and NPS. Consistently in study (IV), alpha-PVP represented the most commonly found NPS while pregabalin was the most commonly abused prescription drug. Buprenorphine abuse appeared minimal (3%), indicating the success of opioid maintenance treatment (Figure 8).

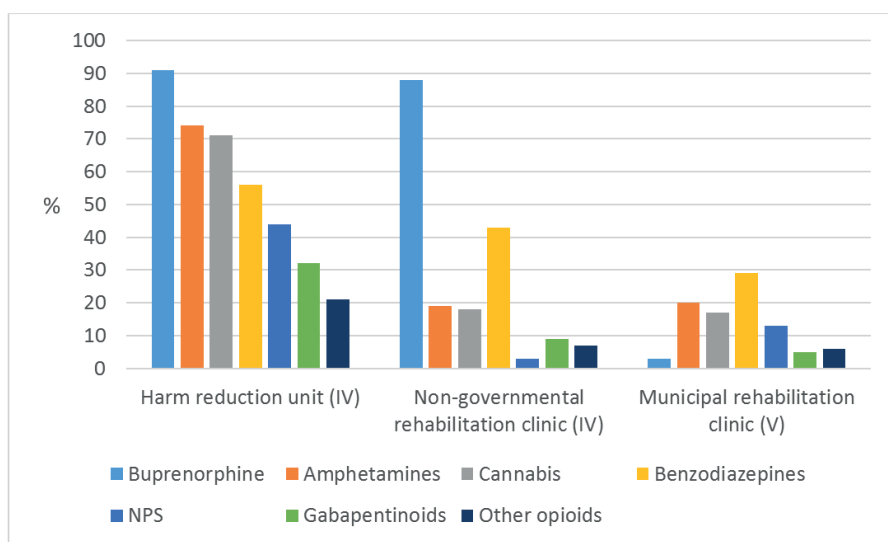


Figure 8. Drug abuse rates (%) for six drug groups and buprenorphine (separated from other opioids to emphasize its high abuse frequency) for subjects visiting a harm reduction unit (IV) and non-governmental (IV) or municipal (V) treatment facilities.

Drug users were often unaware which specific NPS they were abusing (V). In NPS-positive cases, the majority of subjects reported using only an amphetamine or the NPS MDPV. However, drug screening revealed the presence of NPS alpha-PVP, methiopropamine, and fluorinated designer amphetamines. Only one sample detected the same NPS that the individual reported using. This rather striking observation confirms that these results are in accordance with study (IV) as well as with the findings from a Swedish study. In that study, most of the self-reported MDPV abusers tested positive for alpha-PVP [182]. Again, the likelihood of detecting NPS increased with the increasing number of concurrently detected drugs, suggesting that NPS abuse frequently accompanies multiple substance abuse.

In study (V), multiple substance abuse was evaluated by dividing the positive drugs findings into six groups of abused substances: amphetamines, benzodiazepines, cannabis, opioids, NPS, and prescription drugs. In study (IV), in addition to the aforementioned substance groups, buprenorphine was separated from other opioids to emphasize its high abuse frequency. Thus, the number of concurrently abused drugs is not completely comparable between studies (IV) and (V). In a few cases from study (IV), the number of concurrently abused drugs diminished when more than one opioid was detected.

The drug abuse patterns found in studies (IV) and (V) resemble those observed for fatal drug poisoning cases [2,167] and DUID studies [169-171] in Finland. In general, multiple substance abuse frequently occurs. Similar to the results from studies (IV) and (V), among fatal poisonings the most frequent NPS consisted of alpha-PVP and MDPV [2,167]. Moreover, the most frequent concurrently abused drugs among apprehended drivers included benzodiazepines and amphetamines, while buprenorphine was the main intoxicant in fatal poisonings. As exemplified by the high prevalence of alpha-PVP findings, this drug remains the NPS of choice in Finland despite becoming a scheduled narcotic substance in 2013. A similar trend was observed in Sweden, where alpha-PVP was widely abused for several years after scheduling [182]. Both in Sweden and Finland, the abuse of MDPV diminished after its scheduling in 2010 [172,181], which agrees with the low incidence encountered among drug users (IV and V). The observed high frequency of multiple substance abuse involving pregabalin agrees with other studies reporting significant pregabalin abuse combined with the use of other drugs [22,23]. While opioid maintenance treatment did not completely suppress the abuse of opioids (a 9% positive rate in study (V)), the range of abused drugs increased with a higher prevalence of multiple substance abuse among subjects not receiving substitution treatment for drug dependence (IV).

6 GENERAL DISCUSSION

The ever-changing illicit drug scene compromises the feasibility of traditional drug testing. While suitable for high-volume drug screening within workplace drug testing, immunoassays cover only a fraction of the substances relevant to clinical and forensic toxicology. Such methods remain incapable of detecting most NPS and abused prescription drugs (III). Furthermore, they lack identification reliability due to FP and FN results and suffer from the risk of sample manipulation [196]. Therefore, immunoassays for comprehensive drug testing in forensic toxicology are inappropriate.

In addition, analytical confirmation methods often lag behind the emergence of the newest NPS variants. Maintaining an extensive store of reference standards for NPS remains cost-inefficient due to such fluctuating analytical targets. The necessity of quantifying NPS has been questioned by a group of toxicologists, who list a multitude of issues concerning the measurement of NPS [38]. First, interpreting quantitative NPS results should be performed with great care. Information related to their toxicodynamics, stability, and post-mortem issues involving the redistribution and site sampling dependence remain inadequate. Moreover, the validation of quantitative methods is more laborious than that of qualitative approaches as required by accreditation agencies. Given these facts, the group suggested that the qualitative determination of NPS is sufficient for forensic purposes. Furthermore, it has been suggested that using the minimum required performance limit—that is, the lowest analyte concentration detected and confirmed—could provide results that are defensible in court [197].

The development of a multitarget drug screening method involves several analytical compromises allowing coverage of a wide range of analyte lipophilicities and differences in chromatographic behavior. An ideal screening method would also provide a wide dynamic range to detect drugs that may exhibit low concentrations or extremely high levels encountered in drug intoxications. All possible selectivity is required to avoid both FP and FN. This can be achieved using an efficient and generic sample preparation, optimizing UHPLC for resolution rather than speed, applying HRMS, and acquiring additional data on isotope and metabolite patterns and mass fragmentation. Generic sample preparation is useful, particularly within forensic toxicology, where typical clinical sample matrices such as plasma and urine are not always available. Montenarh et al. [97] described a single work-up procedure allowing for the detection of more than 100 drugs in eight matrices. In addition to urine samples, the drug testing procedure from study (I) applies to whole blood, vitreous humor [198], and hair [199] samples.

HRMS screening provides substantial advantages compared to screening based on LRMS. Using HRMS, even co-eluting isomeric compounds can be identified without baseline separation (II), contrary to LRMS in which chromatography may play a more significant role. Even the formula-based tentative identification of unknowns becomes feasible based on an accurate mass at a high resolution (I),

although a reference standard is required for definitive confirmation. When the identification power of a drug screening method reaches the confirmation level, the former two-step practice of screening and confirmation can be diminished to a single step. Omitting that initial step saves time, labor, and expenses. HRMS enjoys a long history in science, involving sophisticated and expensive research instrumentation. Only recently, however, has TOF-MS technology reached a sufficiently high resolution to be considered an HRMS technique. HRMS performed with benchtop TOF-MS and orbitrap techniques is likely to become the gold standard for nontargeted screening owing to its flexibility, sensitivity, and selectivity [7,78,112].

The present method (**I**) relies on DIA with post-targeted data analysis allowing for the handling of moderate-sized datasets. Nontargeted data analysis handles larger and more complex mass spectral datasets. The inclusion of endogenous biomolecules, common impurities, and drug artifacts into the spectral library can be used to assist substance identification [111,135,200]. However, multiple sophisticated tools for processing unbiased data can be exploited to reduce the background noise and leave only that data most likely associated with the compounds of interest. Several noteworthy methodologies that simplify datasets include background noise subtraction [142,201], common fragment search [175,202], mass defect filtering [175,203], and the application of filters and *in silico* fragmentation matching [146]. A true nontargeted approach would identify unknown unknowns. Yet, even with the application of sophisticated tools for data processing, the nontargeted approach remains too laborious for routine casework. The DIA approach using the post-targeted database search presented in study (**I**) enables the identification of hundreds of known unknowns, with the possibility of nontargeted data mining in special cases.

Clinicians would likely embrace a modern HRMS technique for drug testing, since it enables cost-effective analysis with a quick turnaround time. A multidrug screening method would facilitate optimal treatment for acute intoxications by reducing unnecessary supervision and costly treatments [204]. Comprehensive drug testing would enable discrimination between drug-induced psychosis and mental illness, for instance, when a young patient presents with new-onset psychosis or agitated delirium [32]. Knowing the exact drug would also assist the clinician by providing better information to the patient before discharge [71]. However, for now, the primary investigative tool for clinicians regarding NPS abuse remains patient self-reports [67].

The overall turnaround time of the present method (**I**) remains insufficient for emergency settings. Further adjustments involving automation and minimizing sample preparation are essential to reduce the manual workload. Various approaches for automated solid-phase extraction involve sample preparation on cartridges [134], 96-well plates [147,149], and in pipette tips [109]. Dilute and shoot methods involving no sample pretreatment can be applied to reduce analysis time [145,152,190]. An automated online extraction using turbulent flow chromatography could be employed to reach a better LOI than that achieved by dilution alone

[136,205]. The sensitivity, however, might remain inadequate for certain drugs of abuse occurring at low concentration levels [205].

Analytically confirmed epidemiological data on drug abuse in Finland remain scarce. The work in this thesis introduced a new tool for the detection of drug abuse, which can also be applied to extrapolate drug abuse patterns in the general population more reliably than through surveys alone. The typical Finnish drug user frequently abuses multiple substances predominantly consisting of prescription drugs, cannabis, and stimulants (**IV** and **V**). In addition, stimulant NPS (MDPV and alpha-PVP) are favored, increasing the risk of sympathomimetic toxidrome due to their additive effect when combined with other concomitantly abused stimulants. The low prevalence of synthetic cannabinoids in Finland may result from the popularity of homegrown marijuana resulting in an abundant supply of cannabis now approaching the European average [6,15,206]. Moreover, the absence of head shops typical in Central Europe may also contribute to the lack of synthetic cannabinoids on the Finnish drugs scene, which is fortunate, since the risk of a patient needing emergency medical treatment is 30 times higher than that accompanying natural cannabis [207]. NPS abuse is no longer restricted to experimental drug users such as clubbers and psychonauts. Instead, prisoners, people who self-medicate, or individuals seeking to improve their performance, as well as established drug users as also shown in studies (**IV**) and (**V**), now use NPS [4,5]. While NPS do not contribute to fatalities as much as conventionally abused drugs [2,167], they do carry serious public health risks due to the lack of past experiences upon which to rely. That is, information on their toxicity, interactions with other xenobiotics, and long-term health effects remain scarce.

The NPS phenomenon requires constant vigilance from analytical toxicologists and health-care providers. In Finland currently, 16% of all deaths undergo a medico-legal cause of death investigation and 12% an all-encompassing toxicological analysis including drug screening by the new method (**I**) allowing for the straightforward recognition of new drugs [2]. The European Union early warning system disseminates information on the emergence and toxicity of NPS within Europe. For example, early warning system alerts on the emergence of new potent NPS opioids, such as U-47700, enabled the timely detection and subsequent reactions concerning these drugs in Finland [177]. One inspiring example of joint efforts stems from the Swedish STRIDA project, which produced valuable analytically confirmed information on NPS and their toxicity and symptoms for dissemination across the country [178,179]. Such co-operation encompasses a university laboratory, the poisons information center, and emergency departments across Sweden. Thus, collaboration among several authorities, including customs, police, law enforcement officials, emergency hospitals, poison centers, health departments, and drug testing laboratories facilitates the rapid identification of NPS, information exchange, law enforcement efforts, and optimal patient care.

This thesis presents and assesses a comprehensive drug testing method with an equally high reliability as a dedicated target analysis. Identification relies on the broadband DIA approach involving an accurate mass of both precursor and product ions, retention time, precursor isotopic pattern, and reverse database search.

Confirmation analysis for positive results is unnecessary for health care purposes, although two separate analyses remain required in forensic cases [208]. In addition to routine drug screening within forensic toxicology, the new urine drug testing method appears well suited in treatment settings for drug abusers in Finland. Annually, thousands of patient samples are analyzed using this method, which received accreditation from the Finnish Accreditation Service. Since the introduction of the original method, corresponding methods have been introduced in Finland and elsewhere. The method (I) revealed approximately 100 different analytes in clinical case samples analyzed in 2015 by the Department of Forensic Medicine at the University of Helsinki. The most common drugs detected consisted of buprenorphine, benzodiazepines, cannabis, methadone, amphetamine, quetiapine, and gabapentinoids. Table 6 lists all those drugs detected in clinical samples in 2015 not prescribed as a part of normal medical treatment in Finland. Such a broad repertoire of drugs clearly indicates the need for a cost-effective testing method beyond the scope of immunoanalysis (III), as well as beyond the typical targeted LC-MS approaches (II).

Table 6. Illicit drugs and metabolites detected in clinical case samples analyzed by the Department of Forensic Medicine in 2015 using the UHPLC-HR-QTOFMS method (I) listed in order of decreasing prevalence.

1. 11-Nor-9-carboxy- Δ^9 -tetrahydrocannabinol (THC-COOH)	22. Phenmetrazine
2. Amphetamine	23. Ethylone
3. Alpha-pyrrolidinovalerophenone (alpha-PVP)	24. Lysergic acid diethylamide (LSD, metabolite)
4. Methamphetamine	25. Para-methoxymethamphetamine (PMMA)
5. Methylenedioxyamphetamine (MDMA)	26. APINACA (5-hydroxypentyl metabolite)
6. Methylenedioxyamphetamine (MDA)	27. Diphenidine
7. Alpha-pyrrolidinoheptiophenone (PV8)	28. Methoxyphenidine
8. AB-FUBINACA (carboxy metabolite)	29. Methylenedioxypropyvalerone (MDPV)
9. Phenazepam (3-hydroxy metabolite)	30. 4-Methylethcathinone
10. Lormetazepam	31. Mephedrone
11. Flubromazolam	32. 5-(2-Ethylaminopropyl)benzofuran (5-EAPB)
12. Benzoyl ecgonine	33. Bromazepam
13. Etizolam	34. Dibutylone
14. Cocaine	35. Fluorophenmetrazine
15. Ecgonine methylester	36. Fluoromethamphetamine
16. Methiopropamine	37. Hydrocodone
17. Pyrazolam	38. Cocaethylene
18. Flubromazepam (hydroxy metabolite)	39. Meta-chlorophenylpiperazine (mCPP)
19. Isopropylphenidate	40. MDMB-CHMICA
20. Ethylphenidate	41. Methoxetamine
21. Alpha-pyrrolidinohexiophenone (alpha-PHP)	42. Psilocin

7 CONCLUSIONS

In general, the typical drug testing approach involves a two-step procedure to first screen using a quick and non-selective immunoassay followed by confirmation of the positive result using an MS-based method. Commercially available immunoassays and established confirmation methods, however, do not cover the entire range of abused drugs. In addition to typical illicit drugs, drug use today also comprises prescription drugs and NPS. In this thesis a comprehensive screening method was developed and validated, based on state-of-the-art UHPLC-HR-QTOFMS instrumentation (I).

The primary components of this HRMS-based method's success lies in both the acquisition of nontargeted DIA information and the reverse database search using advanced data processing. DIA carried substantial advantages over immunoassay (III) and over the DDA method employed using the same instrument (II) in terms of scope and sensitivity. Furthermore, data acquisition using nontargeted method could be exploited when searching for unknown drugs not included in the target database. Thus, this method enabled tentative identification of synthetic cannabinoid metabolites (I). In addition, the use of diagnostic product ions allowed for the identification of co-eluting isomers (II). Owing to the superiority of the developed method, it has largely replaced immunoassay drug screening for the treatment of people who abuse drugs in Finland.

Furthermore, the method was used to assess the drug abuse patterns in various groups of drug abusers (IV and V). The primary drug findings among subjects not receiving treatment included buprenorphine, amphetamines, cannabis, benzodiazepines, and alpha-PVP (IV). The abuse pattern among subjects undergoing drug withdrawal treatment primarily included buprenorphine, benzodiazepine, and occasionally amphetamines (IV). The majority of subjects in opioid maintenance treatment received medicinal buprenorphine (V) and, therefore, appeared to abuse this drug to a lesser extent than subjects in study (IV). Both studies revealed a high frequency of multiple substance abuse. In particular, subjects not receiving treatment and those abusing NPS used multiple drugs (IV). The lower incidence of multiple substance abuse among subjects receiving treatment clearly showed the importance of drug withdrawal and opioid maintenance treatment in reducing multiple substance abuse.

The comprehensive drug testing method used in this thesis appears feasible in forensic and clinical toxicology. In cause of death investigations, accurately identifying those drugs present in a post-mortem sample remains absolutely necessary. In clinical toxicology such detailed information regarding abused drugs is beneficial for the optimal treatment of drug users and in acute drug poisoning cases. A future challenge lies in developing an even simpler workflow for drug testing requiring minimal sample preparation and a short turnaround time. When these requirements are met, HRMS drug screening will also become amenable to emergency toxicology.

ACKNOWLEDGEMENTS

This study was carried out within the Department of Forensic Medicine at the University of Helsinki between 2011 and 2017. Throughout this period, I have had the honor of working with several amazing individuals whose contributions deserve recognition and thanks.

Firstly, I thank my supervisor Professor Ilkka Ojanperä, who provided me with the opportunity to first work as a research assistant in the Laboratory of Forensic Toxicology, and then saw the potential in me to complete the challenge of travelling along the doctoral path and completing this thesis. He supported me and encouraged me during every aspect related to this research project. In particular, his guidance enhanced my skills in the scientific method and process and in writing, and I thank him for this guidance.

Secondly, I thank my other supervisor Doctor Anna Pelander. Her enthusiastic spirit towards research encouraged me to press forward during times of despair. She taught me the practical laboratory and mass spectrometry related skills necessary to complete this project. In addition, I have had the pleasure of her company during several international conferences.

I truly feel privileged to have had the opportunity to work under the guidance of such experts in high-resolution mass spectrometry.

I also extend my appreciation to my German co-authors, Verena Angerer, Melanie Hutter, and Stefan Kneisel, for providing me samples, confirmation analyses, and wise and useful comments during the manuscript preparation (I). In addition, my Finnish co-authors Doctors Kaarlo Simojoki (IV) and Pertti Heikman (V) deserve thanks for making these studies possible within the clinical toxicology setting.

I express my gratitude to the reviewers of this thesis, Professor (Emeritus) Pekka Männistö and Docent Tiina Kauppila, for their constructive comments and suggestions for improvement.

I'd also like to thank those who, while working in the laboratory, completed their PhD: Jenni Viinämäki, Pirkko Kriikku, Margareeta Häkkinen, Elli Tyrkkö, and Terhi Launiainen. Their advice and company during work, several scientific meetings, as well as outside work has been invaluable. In particular, during the final steps in this process, their scientific examples provided me with immeasurable support. Many thanks go to my fellow doctoral students and future doctors Samuel Mesihää and Anna-Liina Rahikainen for their collegiality and support.

I am grateful to all of my colleagues at the Laboratory of Forensic Toxicology. It has been a pleasure to work with each of these individuals throughout this period. In particular, I thank Professor (Emeritus) Erkki Vuori for being an encouraging example of a passionate scientific researcher. I thank Jari Nokua, MSc, for his help with image processing, as well as Docent Raimo Ketola and Doctor Merja Gergov for their advice and fruitful discussions on mass spectrometry.

Acknowledgements

I also acknowledge the financial support that made this work possible, including support from the European Commission and the Orion Research Foundation.

My sincere thanks go to all of my friends for their encouragement and for providing me so many moments filled with laughter and sympathy when most needed. In particular, Anna, Eeva, Hanna A., Hanna L., Kristiina, and Sari: thank you for being there for me from my early adolescence up to now. Your support all these years has been invaluable.

I am most grateful to my family for their love and support. You have encouraged me and believed in me. Special thanks goes to my twin sister Meri, with whom I have always been able to share the good and difficult times. I am incredibly fortunate to have you there for me always. Finally, my warmest thanks go to Hannu and our lovely children. Without you on my side this work would have not been possible. Thank you, Hannu, for your love, encouragement, and support. And, thank you, my dear beloved twins Venni and Liina, for providing me many joyful and sometimes quite hectic moments. You are my world!

Helsinki, September 2017

Mira Sundström

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